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IFNγ response to paternal antigens during pregnancy

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Medical Microbiology and Immunology

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "IFNγ response to paternal antigens during pregnancy" submitted by Karla Cecilia Trejo-Oliver in partial fulfillment of the requirements for the degree of Master of Science.

To my parents, José and Cecilia

Abstract

The fetus is an allograft to the mother because it inherits paternal antigens. Allospecific T cells against paternal antigens may damage the fetus by producing inflammatory cytokines, such as interferon-γ (IFNγ), or by mediating cytotoxic reactions. Previous studies addressing maternal T cell regulation during pregnancy indicate that IFNγ production against pathogen antigens and cytotoxic responses against paternal antigens are suppressed during pregnancy in two different models, but not in another. I have asked if pregnancy primes for or suppresses IFNγ responses against paternal antigens and if pregnant mice can be deliberately immunized against paternal antigens during pregnancy by measuring IFNγ production after immunization with paternal and third party dendritic cells during allogeneic and syngeneic pregnancies. Our results indicate that pregnancy alone did not prime or suppress IFNγ responses against paternal antigens, and that $CD4^+$ and $CD8^+$ cells could be primed during pregnancy to express IFNγ ex vivo against paternal antigens.

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List of Abbreviations

AEC	3-amino-9-ethyl-carbazole
APC	Antigen presenting cell
BSA	Bovine serum albumin
DC	Dendritic cell
ELISPOT	Enzyme-linked immunospot assay
FACS	Fluorescence-activated cell sorter
FasL	Fas Ligand
FBS	Fetal bovine serum
FITC	Flourescein isothiocyanate
H2	Histocompatibility-2
HLA	Human leukocyte antigen complex
ICAM	Intercellular adhesion molecule
IDO	Indolamine 2,3-dioxygenase
IFNγ	Interferon gamma
IL	Interleukin
LFA	Lymphocyte-function associated antigen
LIF	Leukemia inhibitory factor
LT	Lymphotoxin
MHC	Major histocompatibility complex

mHgs	Minor histocompatibility antigens
NK	Natural killer
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PGE	Prostaglandin E
TCR	T cell receptor
TGF	Transforming growth factor
TH	T helper

INTRODUCTION

In 1953, Peter Medawar described the relationship between the mother and the fetus as a highly successful allograft (1). The fetus is an allograft to the mother because it inherits paternal antigens, including MHC antigens, which are genetically different to the mother. There are more than 40 loci determining histocompatibility and the MHC loci are the strongest ones among these in determining graft rejection. Because MHC loci are closely linked, the fetus usually inherits one complete set, called a haplotype, from the mother and one from the father. Strong immune reactions to MHC antigens may damage the fetus. If left uncontrolled, the maternal immune system could recognize, attack, and destroy the fetus (2). However, outbreeding poses an evolutionary advantage and, therefore, there must be mechanisms that protect the fetus from immune rejection.

Immune System

The immune system responds to invading pathogens and cancer cells to protect the body from various insults. The immune response consists of an initial specific recognition of the invading organism, followed by an effector phase in which the organism is neutralized or eliminated. During the specific recognition phase, the immune system can distinguish between self and foreign organisms. After the initial recognition phase the stimulated immune cell undergoes mitosis and develops a clone of cells with the same antigenic specificity as the original parent cell. This process is called clonal expansion. The effector phase is modulated to suit the particular neutralization and elimination of the invading organism and may be divided into two branches: cellular

immunity, mediated by T cells, and humoral immunity, mediated by antibodies. T cells recognize antigens only when they are presented on the surface of an MHC protein. T cells do not normally respond to self-antigens in association with self-MHC molecules. Therefore, the T cell repertoire is self-tolerant. This is possible due to a selection process that T cells undergo in the thymus in which T cells that are self-MHC restricted are selected (positive selection) and autoreactive T cells are eliminated (negative selection). This process results in the proliferation, survival, maturation, and migration to periphery of T cells that are self-MHC restricted and self-antigen-tolerant.

MHC proteins

MHC proteins are expressed on the surfaces of most cells and are the products of highly polymorphic genes. These proteins are the principal determinants of graft rejection when MHC proteins differ between individuals. Human MHC haplotypes comprise MHC class I, class II, and class III. MHC class I and MHC class II genes code for proteins that present antigens to T cells. Antigens coming from outside the cell are generally presented by MHC class II, while intracellular antigens are generally presented by MHC class I. Products of the MHC class I genes are expressed on almost all cells of the body. Products of MHC class II genes are expressed primarily by professional antigen presenting cells, which include B cells, macrophages, and dendritic cells. The type of MHC that presents the peptide determines which type of T cell will respond. $CD4^+$ T helper cells react in large numbers to MHC class II presentation and $CD8^+$ cytotoxic T lymphocytes react in large numbers to MHC class I presentation. In humans, the MHC class I region is divided

into two gene families: classical, highly polymorphic, constitutively expressed HLA A, B, C, and non-classical, with limited polymorphism, and with expression restricted to certain tissues HLA E, F, G (3). In mouse, the MHC encoding region is a multi-gene cluster comprising MHC class I, MHC class II, and MHC class III genes and it is called histocompatibility-2 or H-2. The individual genes in the MHC class I are designated H2-K, H-2D, and H-2L according to the mouse strain in which they were first identified. MHC class II regions are called I-A and I-E and encode the I-A and I-E MHC class II molecules. There are also non-classical, less polymorphic murine MHC genes such as Qa-1 and Qa-2 (3). In mouse the MHC alleles of a particular strain are designated by a lower case letter. For example, the allele of the K gene in a strain of the b-type is designated K^b (K of b), and the I-A allele of the b-type mouse is designated $I\text{-}A^b$ (I-A of b).

The normal T cell repertoire possesses a high frequency of precursor cells capable of reacting to MHC molecules (4;5). It has been shown that, if not suppressed, potent allogeneic responses against paternal antigens can destroy the fetus (2).

Minor histocompatibility antigens (mHags)

These antigens were originally defined in mice as histocompatibility non-MHC antigens that could induce a rejection response against skin grafts and tumors. Minor antigens are short, MHC-bound peptides derived from endogenous proteins with some degree of polymorphism. Therefore, they are recognized primarily by T cells and are poor at eliciting antibody responses. Any protein that exhibits polymorphism may give

rise to minor antigens. More than 50 loci for minor histocompatibility antigens have been described and include autosomal, H-Y, and mitochondrial antigens. Some minor antigens exhibit immunodominance. That is, when grafts are exchanged between individuals that are genetically different, but MHC-matched, there are many minor histocompatibility differences but T cell responses are limited to a small number of immunodominant epitopes (6). Most, but not all, minor antigens are ubiquitously expressed (7).

T cells specific for minor Hags are present in low frequency, but the effect of T cell responses against several mHags can be very potent and together can be as strong as anti-MHC responses (8). In clinical settings, when the allograft and host are matched for MHC antigens, but mismatched for minor histocompatibility antigens, allogeneic mHags are capable of inducing vigorous immune responses leading to graft rejection and graft-versus-host-disease (7 ; 9).

Antigen presenting cells

The display of peptide/MHC complexes in a form that can be recognized by a T cell is called antigen presentation and cells that display antigens in this form are called antigen presenting cells (APCs). Professional APCs include B cells, macrophages, and dendritic cells. The most efficient APCs are the dendritic cells, which are a heterogeneous population of cells (10). Immature dendritic cells ingest antigen in peripheral tissues, and generate MHC/peptide complexes for presentation to T cells (11). During maturation they migrate to lymphoid organs appropriate for antigen presentation to T cells, such as lymph nodes, and as mature cells they interact and stimulate naive T

cells (12). They are the only cell type that can initiate an immune response in vivo and *in vitro* (13).

Dendritic cells can modulate T cell responses pushing them towards tolerance or immunity depending on the manner of antigen binding and internalization (14), expression of their cell surface molecules, and cytokine secretion (15;16). They can upregulate their co-stimulatory molecules (LFA-3/CD58, ICAM-1/CD54, B7-2, CD86, DC-SIGN) (17) and possess large amounts of cell surface MHC/peptide complexes on their surfaces (18;19). Although MHC class I presentation is usually restricted to antigens that come from inside the cell, dendritic cells can initiate cytotoxic responses through MHC class I presentation of exogenous peptides. Cytotoxic responses against antigens that are not synthesized by the dendritic cell may be advantageous under some circumstances. This process is called cross-presentation (20)((21;22)(20-22).

DCs can promote Th1 or Th2 responses depending on the cytokine environment in which they mature. Dendritic cells can promote Th1 responses when exposed to IL12 at the time of the induction of maturation, or they can promote a Th2 response if they are exposed to PGE-2 at the time of induction of maturation (23;24). Mature dendritic cells become unresponsive to IL12 or PGE-2 (25). Dendritic cells that promote Th1 responses secrete IL12 (26;27;27-29). T cells can also influence dendritic cell behavior (27;30) and survival (31). Therefore, their communication takes place in both directions.

Dendritic cells can induce immunologic tolerance. It has been shown that dendritic cells under certain conditions can prolong allograft survival in vivo (32) and induce $CD4^+$ T cell anergy *in vitro* (33). They can also induce T cell tolerance to peptides derived from apoptotic bodies (34).

T cell recognition of foreign antigens

T lymphocytes are among the components of the immune system that can damage the fetus. They recognize specific antigenic determinants and are responsible for cell mediated immunity and the production of cytokines. $CD4^+$ and $CD8^+$ T cells can damage the fetus by producing inflammatory cytokines (T helper cells), such as IFNγ, or by direct cell to cell killing (cytotoxic T cells). T cells do not recognize soluble proteins but recognize antigens through the T cell receptor only when presented in the context of MHC on an antigen presenting cell. The T cell receptor recognizes portions of the protein presented by MHC and portions of the MHC itself.

T cell differentiation

T cell populations include naïve, effector, and memory cells.

Naïve cells are resting cells that have not encountered antigen. These cells can only be activated by dendritic cells. After activation, a naïve cell will initiate a primary response. About 1 in 10^5 naïve cells is specific for any given antigen. Naïve T helper cells do not secrete IFNγ (35).

Effector cells are T cells that have been activated about 5-7 days and are already specialized to carry a particular function, such as cytotoxicity or secretion of cytokines (e.g. IFNγ) (36). These cells have high expression of cell-adhesion and costimulatory molecules. Effector Th cells can be divided into Th1 and Th2 cells.

Memory cells are generated during a primary response from effector cells or from a distinct naïve cell population (37). These cells are long-lived and are usually in a resting state, but they can be activated with less stringent requirements than a naïve population. They can be activated by dendritic cells, B cells, and macrophages, and they usually migrate to the tissue in which they were originally stimulated.

In the experiments presented here, we have measured IFNγ production from effector and memory T cell populations.

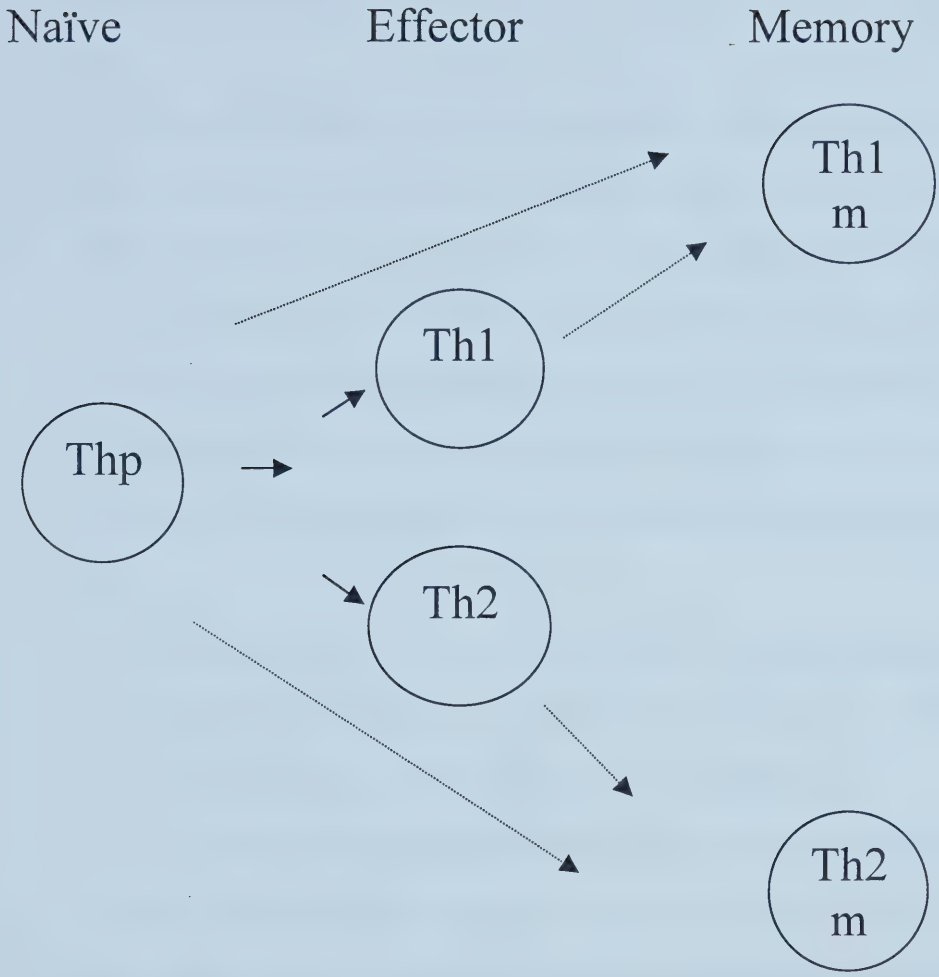

Figure 1.
Diagram of T cell differentiation.
T effector cells differentiate from naïve precursors that have not encountered antigen and do not secrete IL4 or IFNγ. Memory cells are generated during a primary immune response or from a distinct naïve cell population.

Protective mechanisms

The maternal immune system and the fetus have developed a variety of local and systemic mechanisms to protect the fetus from maternal immune attack. Local protective mechanisms at the maternal fetal interface include restricted passage of molecules to and from the fetus (38), altered expression of MHC molecules (39), expression of FasL (40), secretion of hormones that promote fetal growth (41), and regulation of tryptophan metabolism (2). Systemic maternal mechanisms of protection include Th effector specific suppression (42), and suppression of cytotoxic responses against paternal antigens (43) (44).

The experiments presented in this thesis examine systemic suppression of anti-paternal responses during pregnancy, therefore, I will review previous studies that deal with the systemic suppression of T cell responses during pregnancy.

Systemic immune responses can be examined by measuring responses from spleen. The spleen is a large lymphoid organ specialized to trap antigens that are blood-borne. These antigens are carried into the spleen by the splenic artery. In this way, cells found in the spleen can respond to systemic infections and foreign antigens. As antigen enters into the spleen it is trapped by dendritic cells, which then present it to T cells that circulate daily through that site.

Systemic Th effector specific suppression

In order to be effective, the immune system has to choose appropriate effector functions suited for the elimination of a particular pathogen. T helper cells play an important role in the regulation of immune responses by secreting cytokines that direct the growth, differentiation, and effector function of a wide variety of immune cells. They are activated in response to antigenic stimulation and produce protein messengers called cytokines. Upon activation, one T cell can produce a number of different cytokines which are small regulatory polypeptides that act in an autocrine or paracrine fashion. Cytokines bind to specific receptors on the surface of target cells and act synergistically or antagonistically to direct proliferation, differentiation, and function of T cells, B cells, macrophages, and other cells.

Cytokines are important signaling molecules in the reproductive tract. Their secretion is carefully modulated during pregnancy to accommodate an allogeneic fetus. Production of cytokines that promote fetal growth and differentiation is enhanced, while production of cytokines that are potentially detrimental to the fetus is reduced (41).

Based on their distinct cytokine pattern, T helper cells can be divided into different subsets, of which the most extensively described are the type 1 and type 2 (45).

Th1 cells secrete inflammatory cytokines such as IFNγ, IL2, and LT and promote cell-mediated immunity and inflammation (46). Th1 cytokines can damage the fetus (47). Th2 cells secrete IL4, IL5, IL6, IL10, and IL13 that promote humoral immunity. Th2 cytokines do not seem to damage the fetus and in some cases seem to be enhanced during

pregnancy (48). Th1 and Th2 responses inhibit each other: IFNγ inhibits the proliferation of Th2 cells (46), while IL-10 inhibits Th1 cytokine production (49).

The differentiation of T helper cells into Th1 and Th2 subsets is influenced by a variety of factors, of which the most important are the types and amounts of cytokines present in the vicinity of the T cell at the time of differentiation. Th1 and Th2 cells develop from a common IL2 secreting precursor and it is not known if there are intermediate steps in this differentiation process. After *in vitro* or *in vivo* stimulation, small precursor $CD4^+$ T cells develop or differentiate into large effector cells capable of producing IFNγ (Th1 cytokine) or IL4 (Th2 cytokine) after restimulation. Some of the effector cells return to a resting state and can be re-activated by antigen exposure to produce large amounts of cytokines. These cells are referred to as memory cells.

IFNγ: a Th1 cytokine

IFNγ is a Th1 cytokine which mediates inflammation and is released from NK cells and T lymphocytes. It inhibits the proliferation of Th2 cells and promotes the differentiation and activation of macrophages (50) and NK cells. IFNγ also mediates anti-tumor and anti-microbial reactions by inducing the production of TNF and reactive oxygen intermediates from macrophages (51).

High doses of IFNγ are damaging to the fetus and can cause the death of the feto-placental unit (52). If IFNγ is administered in large doses during pregnancy, it leads to increased frequency of resorptions (47). Imbalances in IFNγ levels during pregnancy may

also contribute to the development of recurrent spontaneous abortions (53;54) and preeclampsia in humans (55;56).

Bias away from Th1 immunity during pregnancy

One mechanism that may protect the fetus from damage caused by IFNγ is a bias in the cytokine pattern away from Th1 responses during pregnancy (41). Consistent with this hypothesis, IFNγ production was reduced in syngeneically pregnant mice during Th1-mediated anti-parasite response (42). Pregnancy rendered normally resistant mice susceptible to infection with Leishmania major and there was a bias in the cytokine production away from protective Th1 responses. This susceptibility was correlated with a reduced production of IFNγ in the spleen and lymph nodes and an increase in the footpad parasite load. Therefore, there seems to be a bias away from systemic Th1 responses.

This biased response may explain the decreased susceptibility to cell-mediated autoimmune disorders during pregnancy. Pregnancy is associated with the improvement of rheumatoid arthritis in more than 70% of patients (48;57;58) and with the improvement of multiple sclerosis (59). This may also explain the increased susceptibility to infectious diseases caused by intracellular pathogens such as leprosy, malaria, and leishmaniasis during pregnancy (60). The systemic shift away from Th1 responses during pregnancy may be caused by increased secretion of Type 2 cytokines (IL4 and IL10) (42). In the Leishmania-infection experiments, there was an increased production of Th2 cytokines in spleen and lymph nodes of pregnant animals (42;61).

Local inhibition of Th1 responses may be due to type-3 cytokines (TGF-β) (62) and progesterone. TGF-β inhibits cell-mediated immunity and is found in placenta, decidua, and uterine epithelium (63). Progesterone increases dramatically during pregnancy and induces the differentiation of human T cells into the Th2 subset and transiently induces the production of IL4 from Th1 established clones (64).

Autoimmune diseases caused by Th2 type immunity, such as lupus erythematosus, can flare up during pregnancy (48). This may be due to an increase in Th2 cytokines and a decrease of TH1 cytokines, but the data regarding Th2 type of autoimmune diseases during pregnancy disease is variable.

Suppression of T cells specific for paternal antigens

Previous studies have investigated the modulation of T cells specific for paternal antigens during pregnancy by measuring cytotoxic activity from these T cells.

Fetal antigens may be presented to cytotoxic T cells by maternal or fetal antigen presenting cells in the context of MHC class I. Cytotoxic cells can lyse the target fetal cell using several mechanisms that include perforin mediated and Fas-mediated pathways (65).

Some, but not all studies suggest that these responses are suppressed during pregnancy. In normal mice, anti-paternal cytotoxic T cell responses were not induced by pregnancy. However, the response could be deliberately induced during pregnancy by injection of paternal strain cells, indicating that cytotoxic T cells were present and capable of responding (66). In contrast, two studies using TCR transgenic models

suggested specific immunosuppression against paternal antigens. If maternal $CD8^+$ T cells expressed a transgenic TCR specific for a paternal MHC antigen, levels of the clonotypic TCR were reduced on the transgenic T cells, and tumor grafts bearing the paternal antigen were accepted by pregnant mice (43). Therefore, maternal $CD8^+$ T cells were able to recognize paternal alloantigens specifically, and this recognition resulted in phenotypic changes that prevented the rejection of tumors bearing paternal alloantigens. This lack of rejection was reversed after delivery (43). In a second study, the number of transgenic $CD8^+$ T cells specific for a paternal alloantigen decreased during pregnancy and remained low post-partum. The remaining cells were unresponsive to antigenic stimulation (44). Thus unlike the cytotoxicity responses in normal pregnant mice, these results suggest that pregnancy induces a transient state of tolerance to paternal antigens in transgenic mice. Therefore, cytotoxic responses against paternal antigens seem to be suppressed in some models and not in others.

Rationale

During normal pregnancy, in the absence of infections, $CD4^+$ and $CD8^+$ T cells specific for paternal alloantigens are potentially a major source of IFNγ production that could harm the fetus. Previous studies showed that in an anti-parasite Th1 response IFNγ levels in spleen and lymph nodes were reduced in pregnant mice compared to non-pregnant mice. This suggests that during pregnancy, Th1 IFNγ responses may be suppressed in a non-antigen specific manner. This was explained in terms of a model in which Th1 suppression takes place during pregnancy in order to accommodate an allogeneic fetus. However, these studies were done using syngeneic pregnancies, therefore no allogeneic anti-paternal MHC responses were measured.

Anti-paternal T cell responses were measured in several other studies using transgenic mice. These experiments examined $CD8^+$ T cell cytotoxicity and tumor rejection of cells bearing paternal alloantigens and found that both functions were suppressed during pregnancy. However, no IFNγ responses against paternal antigens were measured in these studies.

Therefore, we have examined the ability of the maternal immune system to mount $CD4^+$ and $CD8^+$ T cell IFNγ responses against paternal antigens. From the Leishmania infection studies, it would be expected that IFNγ would be suppressed generally in a non-antigen-specific manner. From the cytotoxicity studies it would be expected that IFNγ against paternal antigens would be suppressed in an antigen-specific manner, at least for

$CD8^+$ response, because cytotoxicity and IFNγ production are both effector functions of $CD8^+$ T cells.

Hypothesis and objectives

We hypothesized that IFNγ responses against paternal antigens are suppressed during pregnancy to protect the fetus from damage caused by inflammatory responses promoted by this cytokine.

We tested whether pregnancy induces any enhancement or suppression of $CD4^+$ and $CD8^+$ T cell IFNγ responses against MHC alloantigens in the following situations:

1. Natural (cross-reactive?) responses against paternal antigens
1. Natural (cross-reactive?) responses against non-paternal alloantigens
2. Immunization against paternal antigens during pregnancy
3. Immunization against non-paternal antigens during pregnancy

MATERIALS AND METHODS

Mice

6-8 week old MHC congenic mice, C57 BL/10 ($H2^b$), B10.D2 ($H2^d$), and B10.BR ($H2^k$), were obtained from Jackson Laboratory, ME. Mice were housed in the Health Sciences Laboratory Animal Services facility at the University of Alberta (Edmonton, AB, Canada) in accordance with the guidelines of the Canadian Council on Animal Care. C57 BL/10 females were mated with C57 BL/10, B10.D2, and B10.BR males. One male and two females were housed per cage overnight, and females were checked the following morning for the presence of a vaginal plug. The day on which the plug was found was considered to be day 0 of pregnancy. Pregnant females were removed from the mating cages and housed with non-pregnant control mice.

Dendritic cell enrichment

Single cell suspensions of spleen were made in RPMI 1640 medium supplemented with 0.1% 2-mercaptoethanol, 0.05mg/ml gentamycin, and 0.5% heat inactivated syngeneic normal mouse serum. Spleen cells were incubated for 90 minutes, at 37°C. After incubation, non-adherent cells were washed off with warm medium and adherent cells were incubated overnight (16 hours) at 37°C. After the overnight incubation, non-adherent cells, enriched for dendritic cells (but may also include some contaminating B cells and T cells that were not washed off), were harvested, washed and resuspended at 1

spleen equivalent (approximately $0.5x10^6$ dendritic cells)/ 100µl RPMI. It is important to precoat all tubes with medium and normal mouse serum. If this is not done, the dendritic cells will adhere to the tube and will be lost. To obtain the normal mouse serum, mice were bled by cardiac puncture. The serum was separated by centrifugation (7 minutes, at room temperature, at 1500 rpm) and heat inactivated for 15 minutes in a 56°C water bath.

The enriched cell preparations were stained with the anti-CD11c antibody N418, and anti-MHC PE (Pharmingen) and their purity was assessed by FACS analysis. Cell preparations consisted of approximately 50% of dendritic cells.

Immunization and cell separation

Preparation of responding cells:

Pregnant (day 7 of pregnancy) and age-matched non-pregnant mice were injected intraperitoneally with one spleen equivalent (100µl) dendritic cell enriched preparation in RPMI 1640. On day 14 of pregnancy, mice were sacrificed and spleens were harvested. Single cell suspensions of spleen were prepared in RPMI 1640 medium containing 8% FBS, 0.05mg/ml gentamycin, and 0.1% 2-mercaptoethanol. Aliquots of spleen were positively sorted for $CD4^+$ and $CD8^+$ cells using subsaturating amounts of antibodies (anti-CD8 FITC, clone YTS 169.4 and anti-CD4 CyChrome, Pharmingen, clone H129.19). Subsaturating amounts of antibodies were used (0.5 µg/spleen) in order to leave some $CD4^+$ and $CD8^+$ molecules on the surface of the T cells free to interact with the antigen presenting cell.

Preparation of antigen presenting cells

Potential IFNγ secreting cells were removed from the antigen presenting cells in order to measure exclusively IFNγ production by responding cells.

C57BL/10 ($H2^b$), B10.D2 ($H2^d$), or B10.BR ($H2^k$) spleen cells from non-pregnant mice were depleted of $NK1.1^+$, $CD4^+$. and $CD8^+$ cells. Depletion was done using Dynal dynabeads, according to the manufacturer's protocol. Antibodies used for the depletion were anti-CD8-biotin (clone 53-6.7) at 10μg/ml, anti-CD4-biotin (clone H129.19) at 10μg/ml, anti-NK1.1-biotin (clone PK136) at 10 μg/ml.

Fetus and resorptions

On day 14 of pregnancy, the uterus was isolated and the number of viable fetuses as well and the number of resorption sites were counted. Fetal resorptions were identified by their smaller size and the appearance of necrotic tissue when compared with viable fetuses.

ELISPOT

Multiscreen 96 well plates (Millipore Corporation, Bedford, MA) were coated for two hours with 100 μl/well of 4 μg/ml AN18 antibody. After the coating step, the plates were blocked with RPMI 1640 medium containing 8% FBS, 0.05mg/ml gentamycin, and 0.1% 2-mercaptoethanol. Responder cells (unseparated, $CD4^+$, and $CD8^+$ cells) (dilutions

starting at 0.2-2.7 million cells/well) from C57 BL/10 ($H2^b$) mice were added to the plate along with stimulator cells from C57 BL/10 ($H2^b$), B10.BR ($H2^k$), or B10.D2 ($H2^d$) mice at $5x10^5$ cells per well. After a 16 hour incubation on the ELISPOT plate, the cells were removed. The plates were then washed after each incubation with PBS buffer containing 0.05% Tween 20 (Sigma Chemical Co.). The secondary antibody, XMG1.2-biotin (100 μl/well of 2 μg/ml XMG1.2 in PBS containing 1% BSA (Sigma Chemical Co.) and 0.05% Tween 20), was added for 30 minutes. A 30 minute incubation with peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) followed by 15 minute incubation with AEC substrate (Vector Laboratories, Inc., Burlingame, CA) was used to develop the IFNγ spots.

The plates were air-dried and spots were counted under a dissecting microscope.

All counts were expressed as spots per million responder cells

Statistics

Data values were analyzed using the non-parametric Mann-Whitney test. We used a non-parametric test because of the small numbers of mice in each group and to reduce the effect of outliers. We excluded mice that showed very high IFNγ production in the negative controls (syngeneic responses) and no response at all including the response in the positive control (Con A stimulation).

Data for all the figures shown are not normalized to the controls. By normalizing (divide the value for the pregnant mouse by the value for the control mouse used on the same day and multiply this number by the average of the values for that control group) we minimize day to day variation. However, this was not necessary because

normalization did not change the conclusions drawn from the data shown. Pregnant mice were matched to a non-pregnant control used on the same day and the pair was shown as adjacent bars on the graphs. In some cases, there was one control mouse for two pregnant ones.

RESULTS

1. Effects of pregnancy on natural responses to paternal MHC antigens in unimmunized mice

We studied MHC responses because MHC proteins are highly polymorphic in a population and there is a high frequency of T cells that will respond to them, even in naïve, unimmunized individuals. Because there is a background anti-MHC response in unimmunized individuals, there is the potential to detect either an increase or a decrease in the response. Measuring anti-MHC responses in non-immunized mice determines the effects of pregnancy on responses preexisting before pregnancy, as well as possibly responses initiated during pregnancy. This allows us to measure any enhancement, suppression, or ignorance of anti-MHC responses during pregnancy without deliberate immunization. In addition, in normal pregnancies MHC proteins are almost always different between mother and fetus and, therefore, it is biologically relevant to study maternal immune responses against these antigens.

C57BL/10 ($H2^b$) females were mated with C57BL/10, B10.BR, or B10.D2 males, expressing $H2^b$, $H2^k$, and $H2^d$, respectively. These mouse strains are congenic for MHC, that is, they only differ genetically in the MHC Class I and II haplotypes, but are otherwise genetically identical. Thus, the fetuses from these pregnancies were completely syngeneic with the mothers, but differed only in the MHC on one chromosome. This ensured that any maternal allogeneic responses against paternal antigens measured directly *ex vivo* were directed against MHC proteins. We measured IFNγ production in

response to both paternal and third party MHC antigens to distinguish between specific and non-specific alterations of IFNγ production. If regulation of anti-MHC IFNγ production is non-specific, responses to both paternal and third party antigens will be affected in a similar manner. However, if this regulation is specific, only anti-paternal MHC IFNγ responses will be altered. Analysis of potential antigen-specific effects was measured very precisely by comparing paternal and third party responses in the same mouse. This controls for mouse to mouse and day to day variations, and, therefore, is the best comparison for measuring antigen-specific effects.

In order to measure natural IFNγ responses, we used the ELISPOT assay because this measures the potential of individual T cells to secrete IFNγ directly *ex vivo*. As naïve T cell differentiation requires about three days for the acquisition of significant IFNγ-secreting ability (36), the ELISPOT measures the phenotype of T cells that have differentiated *in vivo*, with no differentiation *in vitro*. To stimulate the cells during the 16-hour ELISPOT we used syngeneic, paternal allogeneic, and third party allogeneic spleen cells depleted of cells that can secrete IFNγ ($CD4^+$, $CD8^+$, and NK cells).

Spleen cells were taken from non-pregnant and 14-day pregnant mice that had not been immunized with any alloantigens. *(These mice were injected with syngeneic cells, because they were the control group of a larger experiment to study the effect of immunization, as described below. In previous experiments we did not see a significant difference between non-immunized mice and mice injected with syngeneic cells.)*. We chose day 14 of pregnancy because previous experiments have shown a non-specific IFNγ suppression during mid-pregnancy (42).

Previous experiments have shown that cytotoxicity from $CD8^+$ cells and IFNγ from $CD4^+$ cells are suppressed during pregnancy. Therefore, we wanted to analyze IFNγ production in both $CD4^+$ and $CD8^+$ cells.

IFNγ responses against MHC antigens are easily detectable but variable

Figure 2 shows that non-pregnant mice gave a variable but detectable response against the alloantigens $H2^k$ (median of 24.5 IFNγ spots per million cells) and $H2^d$ (median of 24 IFNγ spots per million cells), but a much lower response on syngeneic APC (median of 3 spots per million cells). This background response against MHC alloantigens is expected because of the high frequency of anti-MHC cross-reactive T cells specific for environmental antigens (4). The differences between the syngeneic ($H2^b$) response and each allogeneic response were significant at the 0.001 level.

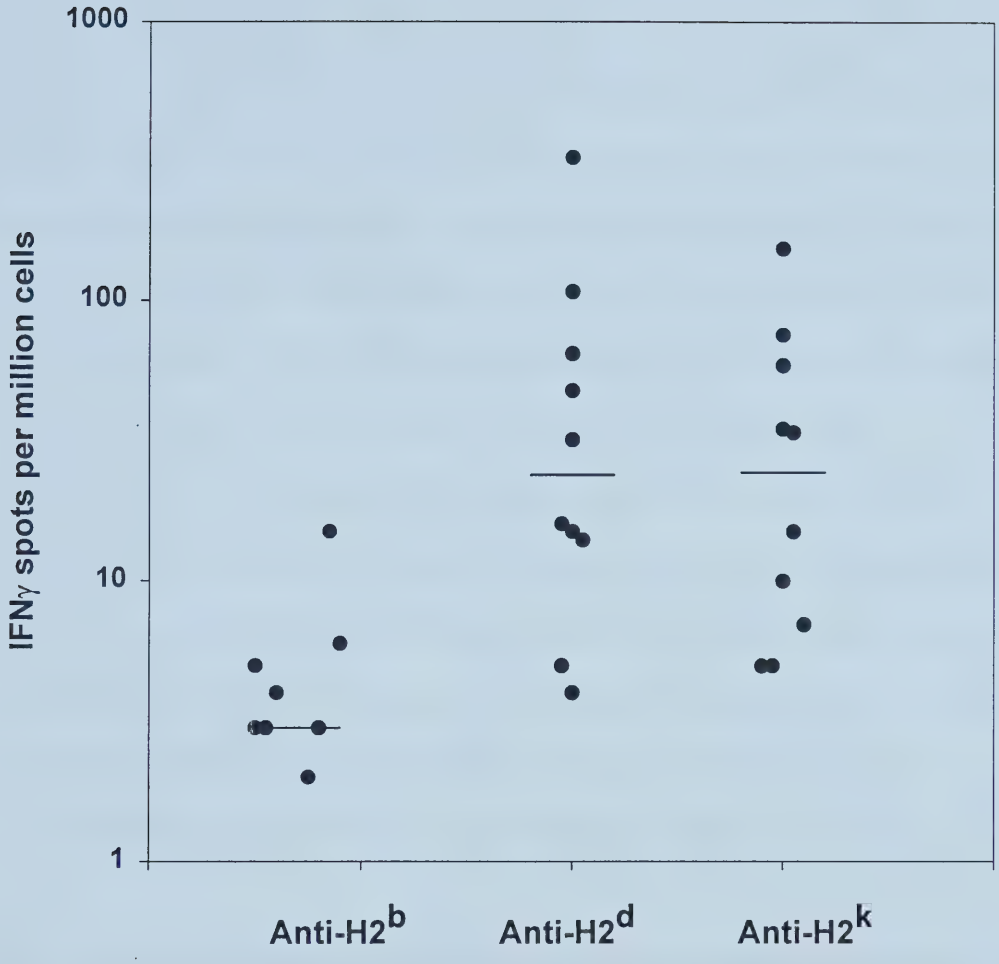

Figure 2.
C57BL/10 ($H2^b$) non-pregnant control mice were injected i.p. with syngeneic dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. After 7 days, unseparated spleen cells were harvested and stimulated *in vitro* with syngeneic ($H2^b$), allogeneic B10.BR ($H2^k$), or allogeneic B10.D2 ($H2^d$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice. Medians are represented by horizontal bars.

There are about 20 million T cells in one spleen and only a portion of these T cells (we assume approximately 10% based on CD44 expression) belong to the effector and memory populations. Allospecific T cells account for approximately 5% of T cells. This means that 0.5% of the T cells in spleen are allospecific effector cells. In our ELISPOT assays an average of 0.005% of the cells from unimmunized mice responded by secreting IFNγ against allogeneic MHC antigens. Therefore 1/100 of the theoretical effector/memory T cells produced IFNγ. Several factors may contribute to the low number of IFNγ producing cells found. For example, only a portion of the population, e.g. 10%, may be Th1/Tc1, and our assay may not be very efficient in reflecting the actual number of Th1 or Tc1 cells, e.g. 10% efficiency. This would result in 1% of the theoretical number. The theoretical number of allospecific effector cells is an approximation because we are assuming the percentage of effector and memory T cells.

IFNγ production in response to allogeneic MHC is similar in syngeneically pregnant mice and non-pregnant controls

Our results in figure 3 show that IFNγ responses against MHC alloantigens that are NOT present on the fetus were not enhanced or suppressed by pregnancy. IFNγ responses against syngeneic H2b (median 0 spots per million cells), allogeneic $H2^d$ (median 3 spots per million cells) and allogeneic $H2^k$ (median 5 spots per million cells) antigens in syngeneically pregnant mice were similar to the responses from matched controls (1.5, 6,

and 10 spots per million cells against $H2^b$, $H2^d$, and $H2^k$ respectively, and p=0.788, 0.323, and 0.621, respectively). This indicates that there is no general suppression or enhancement of IFNγ responses.

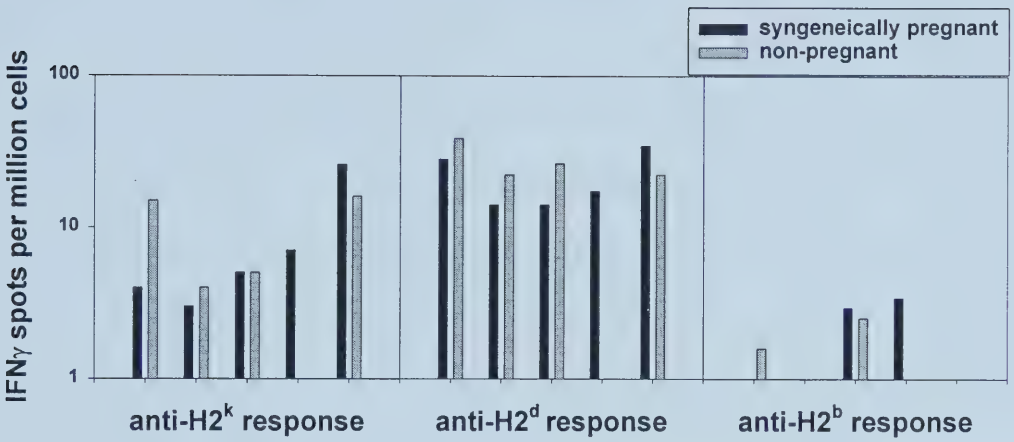

Figure 3.
IFNγ response in syngeneically pregnant and non-pregnant mice.
C57BL/10 ($H2^b$) mice were mated with C57BL/10 males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with syngeneic dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, unseparated spleen cells were harvested and stimulated *in vitro* with syngeneic ($H2^b$), allogeneic B10.BR ($H2^k$), or allogeneic B10.D2 ($H2^d$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice. Adjacent black and gray bars represent pregnant and non-pregnant control mice that were used on the same day. All pregnant mice have a control mice done on the same day. In some cases, there is one control mouse per two pregnant mice. In those cases the bar for the control mouse appears adjacent to the bar of the first mice and the bar for the second pregnant mouse appears by itself.

In figure 4, another comparison that is consistent with this point is that allogeneic pregnant mice with $H2^d$ and $H2^k$ fathers showed similar responses against non-paternal antigens to their matched controls (medians of spots per million cells: 6 and 0.5 vs 3.5 and 1.5 for $H2^b$ antigens, 56 vs 76 for $H2^d$ antigens, and 78.5 vs 108 for $H2^k$ antigens). If pregnancy alone had an enhancing or suppressive effect on IFNγ responses against non-paternal MHC alloantigens, secretion of IFNγ in pregnant and non-pregnant mice would be different. However, the response against non-paternal antigens was similar in the two groups. That is, $H2^b$ mice pregnant with fetuses bearing $H2^d$ antigens showed similar IFNγ responses against $H2^k$ MHC antigens to responses from their non-pregnant control mice, $p=0.657$. Similarly, $H2^b$ mice pregnant with $H2^k$ antigens showed similar IFNγ responses against $H2^d$ antigens to responses from their non-pregnant controls $p=0.714$. This indicates that pregnancy alone did not affect IFNγ responses against alloantigens that were not present on the fetus.

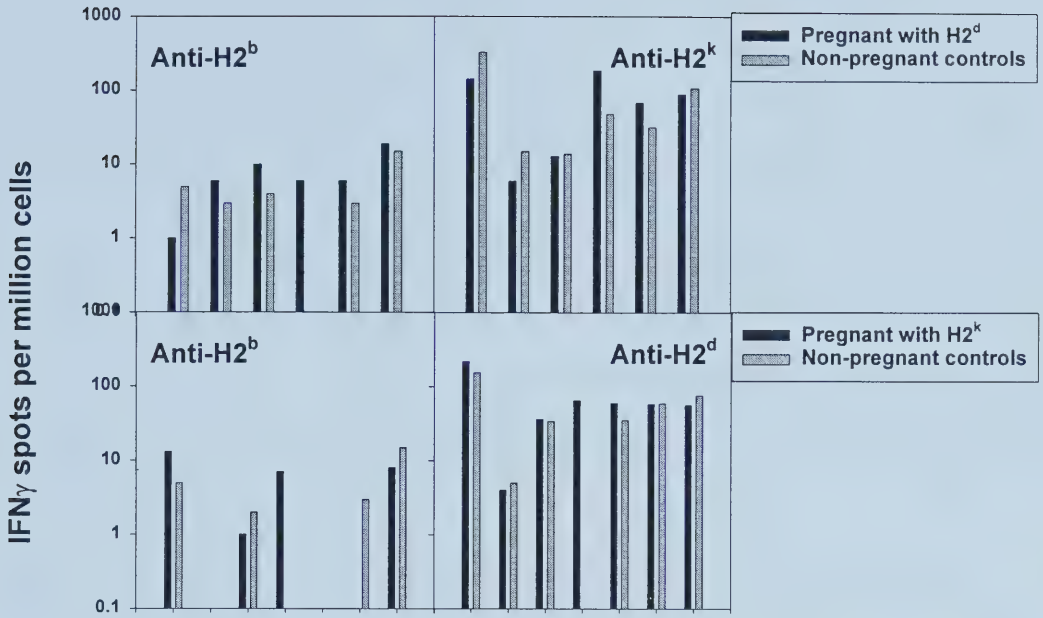

Figure 4.
IFNγ response against non-paternal antigens during allogeneic pregnancy. C57BL/10 ($H2^b$) mice were mated with B10.BR ($H2^k$) or B10.D2 ($H2^d$) males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with syngeneic dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, unseparated spleen cells were harvested and stimulated *in vitro* with syngeneic ($H2^b$), or non-paternal allogeneic ($H2^d$ or $H2^k$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice. Adjacent black and gray bars represent pregnant and non-pregnant control mice that were used on the same day. All pregnant mice have a control mice done on the same day. In some cases, there is one control mouse per two pregnant mice. In those cases the bar for the control mouse appears adjacent to the bar of the first mice and the bar for the second pregnant mouse appears by itself.

However, when compared with allogeneic pregnancies $H2^d$ and $H2^k$, syngeneic pregnancies showed lower IFNγ production in response to MHC alloantigens, $p<0.001$ and $p=0.001$ respectively (compare figures 3 and 4). The reason for this difference is that there was a bias in the responses depending on the day in which the experiment was done. In this experiment, spleen cells from different pregnancies were harvested and challenged on different days. Different antigen presenting cell preparations may be stronger or weaker than others and may explain why some responses were low on certain days and high on others. When comparing values normalized to the control mice used on the same day, there is no statistical difference between allogeneic and syngeneic pregnancies. It is also possible that the mice used for syngeneic pregnancies had low anti-MHC responses due to environmental conditions that we may not be able to control for. Our results show that, in comparison to responses from other days, IFNγ production was low in syngeneically pregnant mice and their matched non-pregnant controls. This emphasizes the importance of the matched non-pregnant controls on each day. When the results are normalized to their matched controls, responses in syngeneic pregnancies did not show lower responses to MHC alloantigens.

The conclusions for other results were not affected by normalization.

Pregnancy does not enhance or suppress IFNγ responses against paternal MHC antigens.

In the previous paragraphs we have discussed a non-specific effect on IFNγ responses against MHC antigens associated with pregnancy, for example effects associated with

hormonal changes. In the next paragraph we will discuss antigen-specific effects on responses against paternal MHC alloantigens.

Our results in figure 5 showed no evidence of specific suppression or enhancement of IFNγ responses against paternal antigens. The anti-paternal MHC responses from allogeneically pregnant mice with $H2^d$ and $H2^k$ fetuses (median of 61.5 spots per million cells for $H2^d$ and 123 for $H2^k$) were similar to the responses against these antigens from non-pregnant matched controls (median of 47 spots per million cells for $H2^d$ and 108 for $H2^k$), p=0.810 and 0.568 respectively.

In addition, pregnant $H2^b$ mice with fetuses bearing $H2^d$ antigens showed similar responses against $H2^d$ (median 61.5) as pregnant mice with fetuses bearing $H2^k$ antigens (median 56), p=0.637 (Figures 4 and 5). Similarly, pregnant $H2^b$ mice with fetuses bearing $H2^k$ antigens showed similar responses against $H2^k$ (median 123) as pregnant mice with fetuses bearing $H2^d$ antigens (median 78.5), p=0.700 (compare figures 4 and 5). This indicates that pregnancy did not selectively enhance or suppress anti-paternal IFNγ responses. However, as a group, responses from allogeneically pregnant mice were higher than the ones from syngeneically pregnant mice. As discussed above, this is due to day to day variations in antigen presenting cell preparations because responses from pregnant mice were similar to the ones from their matched non-pregnant controls, and when comparing normalized values among these groups there is no statistical difference.

(These mice were injected with syngeneic cells, because they were the control group of a larger experiment to study the effect of immunization, as described below. In previous experiments we did not see a significant difference between non-immunized mice and mice injected with syngeneic cells.)

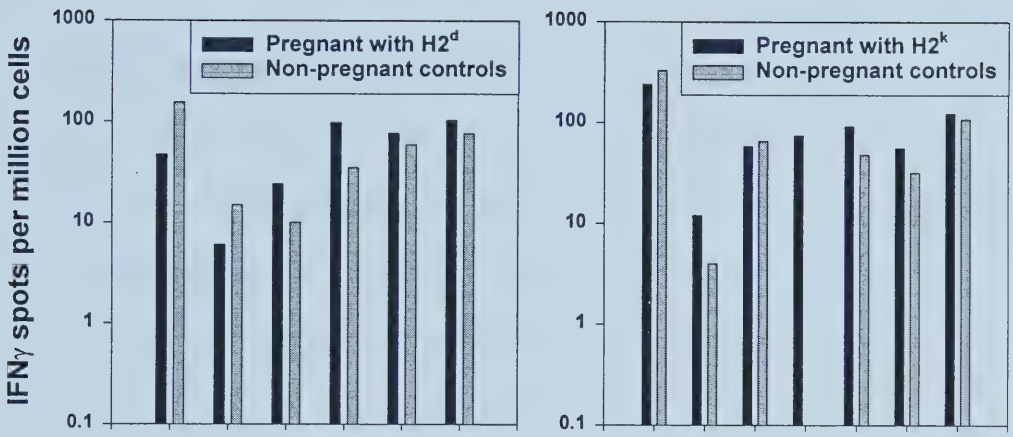

Figure 5.
IFNγ response against paternal antigens.
C57BL/10 ($H2^b$) mice were mated with B10.BR ($H2^k$) or B10.D2 ($H2^d$) males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with syngeneic dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, unseparated spleen cells were harvested and stimulated *in vitro* with syngeneic ($H2^b$), or paternal allogeneic ($H2^k$ or $H2^d$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice. Adjacent black and gray bars represent pregnant and non-pregnant control mice that were used on the same day. All pregnant mice have a control mice done on the same day. In some cases, there is one control mouse per two pregnant mice. In those cases the bar for the control mouse appears adjacent to the bar of the first mice and the bar for the second pregnant mouse appears by itself.

Potential antigen-specific effects measured more precisely with internal controls from the same mouse

Because background anti-allo-MHC responses were variable, we compared IFNγ production against $H2^d$ and $H2^k$ in each mouse using the data from the previous figures. As seen in figure 6, the two responses correlated well in non-pregnant mice suggesting that environmental priming affected both responses to a similar extent. If alloantigens on the fetus could prime maternal anti-MHC responses, then we would expect that a mouse bearing $H2^k$ fetuses should show a selectively higher anti-$H2^k$ than anti-$H2^d$ response. Similarly a mouse bearing $H2^d$ fetuses should show selectively higher maternal anti-$H2^d$ than anti-H2k response. No evidence for this is apparent in figure 6, $p=0.248$. Thus, even using the sensitive comparison of two MHC responses within the same mouse, no maternal enhancement or suppression of the natural anti-paternal responses was detected.

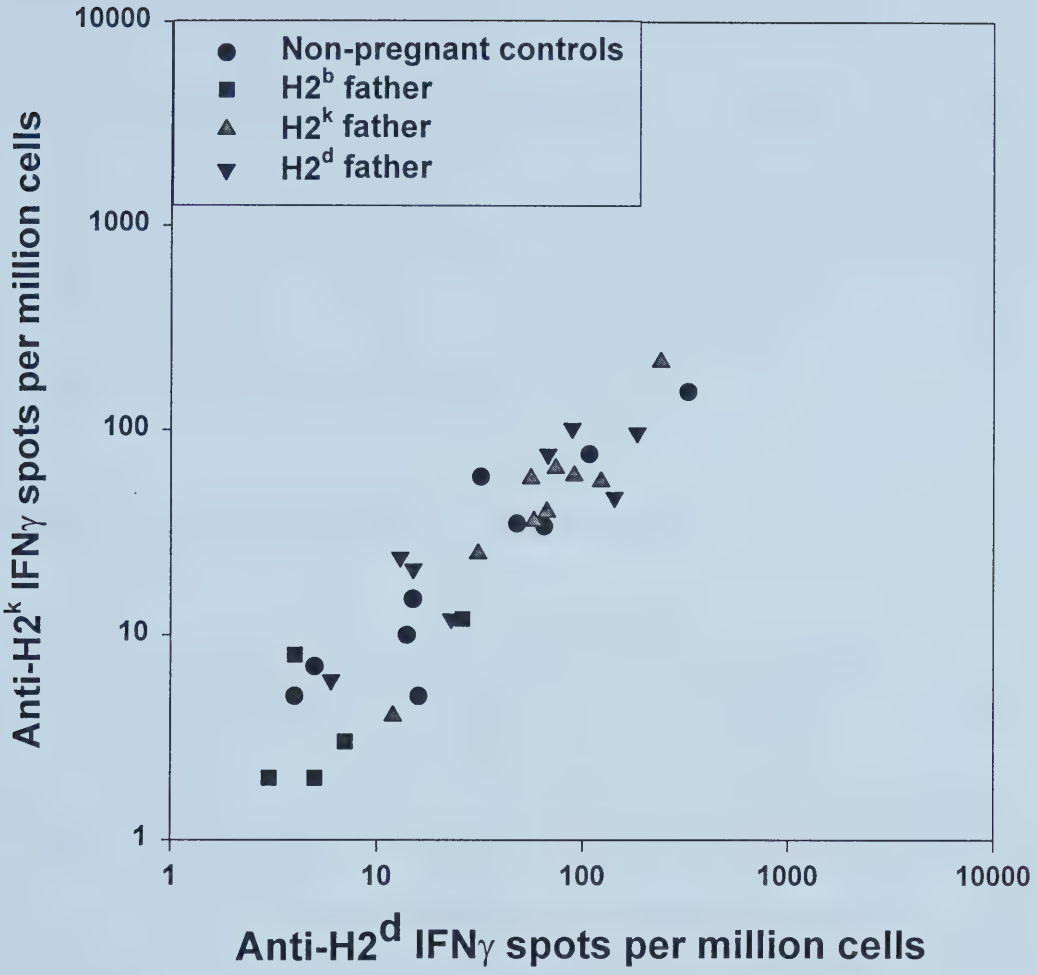

Figure 6. Comparison of IFNγ response against $H2^k$ and $H2^d$ antigens in the same mouse.

C57BL/10 ($H2^b$) mice were mated with C57Bl/10 ($H2^b$), B10.BR ($H2^k$) or B10.D2 ($H2^d$) males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with syngeneic dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, unseparated spleen cells were harvested and stimulated *in vitro* with allogeneic B10.BR ($H2^k$) or allogeneic B10.D2 ($H2^d$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice. This data was shown in figures 2-5.

2. Effects of pregnancy on induction of responses to MHC alloantigens

In the previous section we examined background responses that were initiated before or during pregnancy. In this section we want to study responses initiated during pregnancy. To examine whether pregnancy has any effects on anti-MHC IFNγ responses initiated during pregnancy, we deliberately immunized pregnant mice with a mixture of two different allogeneic dendritic cell preparations.

Induction of IFNγ allogeneic responses with dendritic cells

To establish conditions for priming anti-MHC responses, non-pregnant C57 BL/10 ($H2^b$) mice were immunized intraperitoneally with dendritic cells from congenic B10.Br and B10.D2 mice, expressing $H2^k$ and $H2^d$, respectively. Optimization experiments showed that 0.5 spleen equivalents induced an easily measurable anti-MHC response. We decided to immunize with one spleen equivalent to guarantee a response. After a 7 day period, spleen cells were harvested and stimulated with syngeneic, B10.BR, and B10.D2 spleen cells depleted of $CD4^+$, $CD8^+$, and $NK1.1^+$ cells. The number of IFNγ secreting cells was measured by ELISPOT.

Unimmunized animals showed no IFNγ production in response to syngeneic stimulation and a low frequency of cells producing IFNγ in response to $H2^k$ or $H2^d$ stimulation, consistent with other studies (67) and the results in the first section of this thesis. Immunization with $H2^k$ induced a 37 fold increase in the IFNγ anti-$H2^k$ response, but no increase (0.87 fold) in the anti-$H2^d$ response (figure 7). Similarly, immunization

with $H2^d$ induced a 43 fold increase in the anti-$H2^d$ response, but only a 1.6 fold increase in the anti-$H2^k$ response. This indicates that 7 day immunization with allogeneic dendritic cells can induce an easily measurable IFNγ response with low crossreactivity between $H2^k$ and $H2^d$.

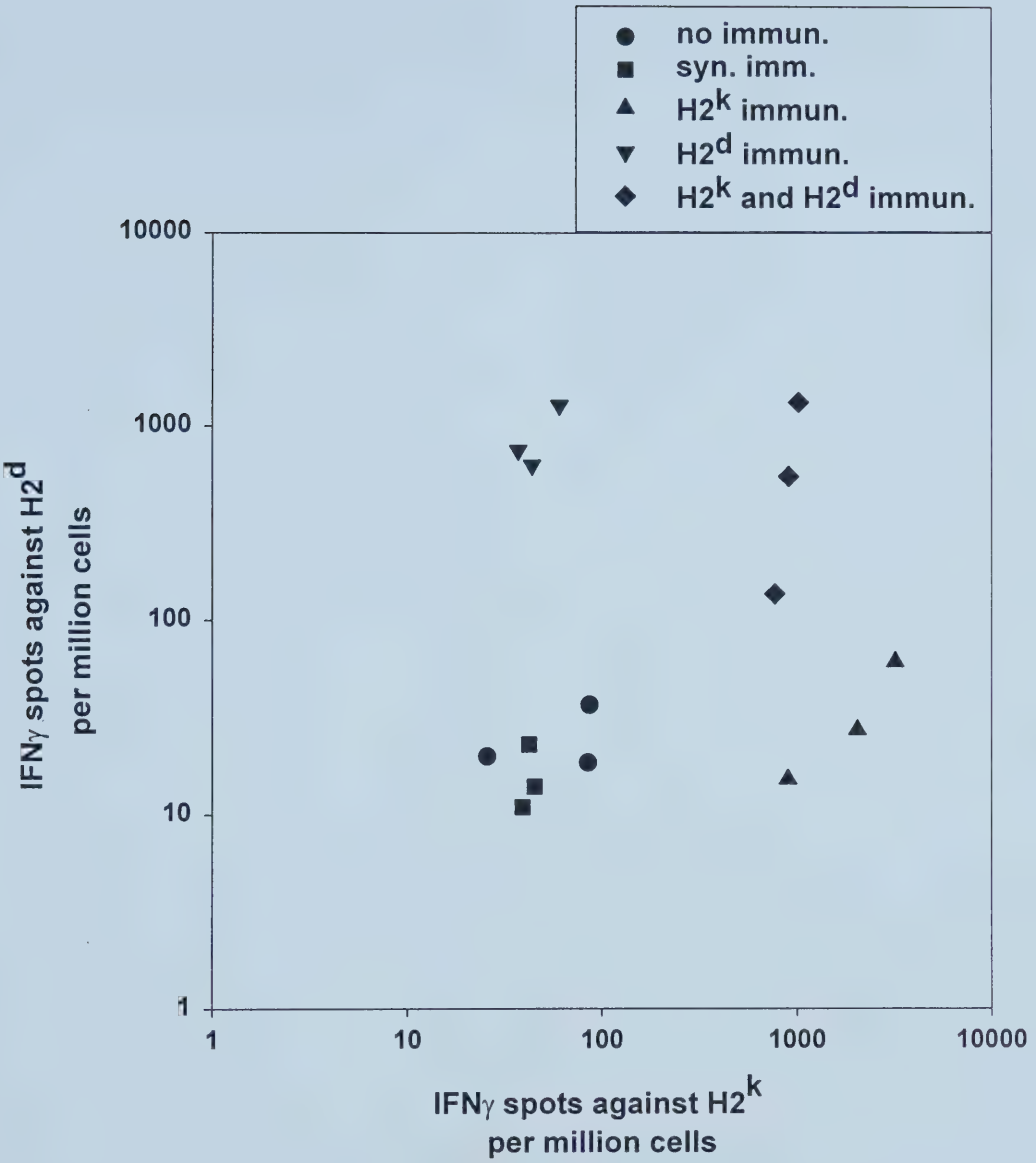

Figure 7.
Immunization with dendritic cells can induce an IFNγ response with low crossreactivity between anti-$H2^k$ and anti-$H2^d$ responses.
C57BL/10 ($H2^b$) mice were were injected i.p. with syngeneic C57BL/10 ($H2^b$), with allogeneic B10.D2 ($H2^d$), with allogeneic B10.BR ($H2^k$), or with both types of allogeneic B10.D2 and B10.BR dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. Seven days after immunization, unseparated spleen cells were

harvested and stimulated *in vitro* with syngeneic, B10.BR ($H2^k$), or B10.D2 ($H2^d$) allogeneic spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice.

An IFNγ response against paternal MHC antigens can be induced during pregnancy.

To test whether fetal antigens influence anti-MHC responses initiated during pregnancy, pregnant mice were immunized at day 7 of pregnancy with a combination of $H2^k$ and $H2^d$ dendritic cells.

C57BL/10 ($H2^b$) females were mated with C57BL/10, B10.BR, or B10.D2 males, expressing $H2^b$, $H2^k$, and $H2^d$, respectively. Pregnant and matched non-pregnant control mice were injected intraperitoneally with one spleen equivalent of a preparation of cells enriched for dendritic cells. Dendritic cells were derived from MHC congenic B10.BR ($H2^k$) and B10.D2 ($H2^d$) mice in order to elicit responses against MHC antigens alone. We used dendritic cells to induce an IFNγ response because they are potent antigen presenting cells and are the only cell type able to stimulate an immune response from naïve T cells. Therefore, they may induce proliferation and differentiation of naïve allospecific cells or proliferation of memory allospecific cells. Immunization with both types of allogeneic dendritic cells provided a sensitive measure of potential alterations of anti-paternal and anti-third party responses in the same mouse. To control for the dendritic cell immunization, we injected mice in group 1 with syngeneic dendritic cells.

We chose to immunize on day 7 of pregnancy because that allowed for induction and differentiation of an IFNγ response during mid-pregnancy. As discussed before, mid-pregnancy was chosen because previous experiments using syngeneic pregnancies showed a non-specific suppression of IFNγ responses during this period.

IFNγ secretion from spleen cells against syngeneic, paternal allogeneic, and third party allogeneic cells was measured by ELISPOT on day 14 of pregnancy. Paternal and third party responses allowed us to evaluate antigen specific and non-antigen specific effects of pregnancy respectively.

As shown in figure 8, allogeneic immunization induced a generally higher IFNγ response against both paternal and third party MHC antigens in pregnant mice immunized with allogeneic cells than in pregnant mice immunized with syngeneic cells (compare figure 6 and figure 8), in agreement with the results in figure 7 for non-pregnant mice. $p<0.001$ for both anti-$H2^d$ and anti-$H2^k$ responses.

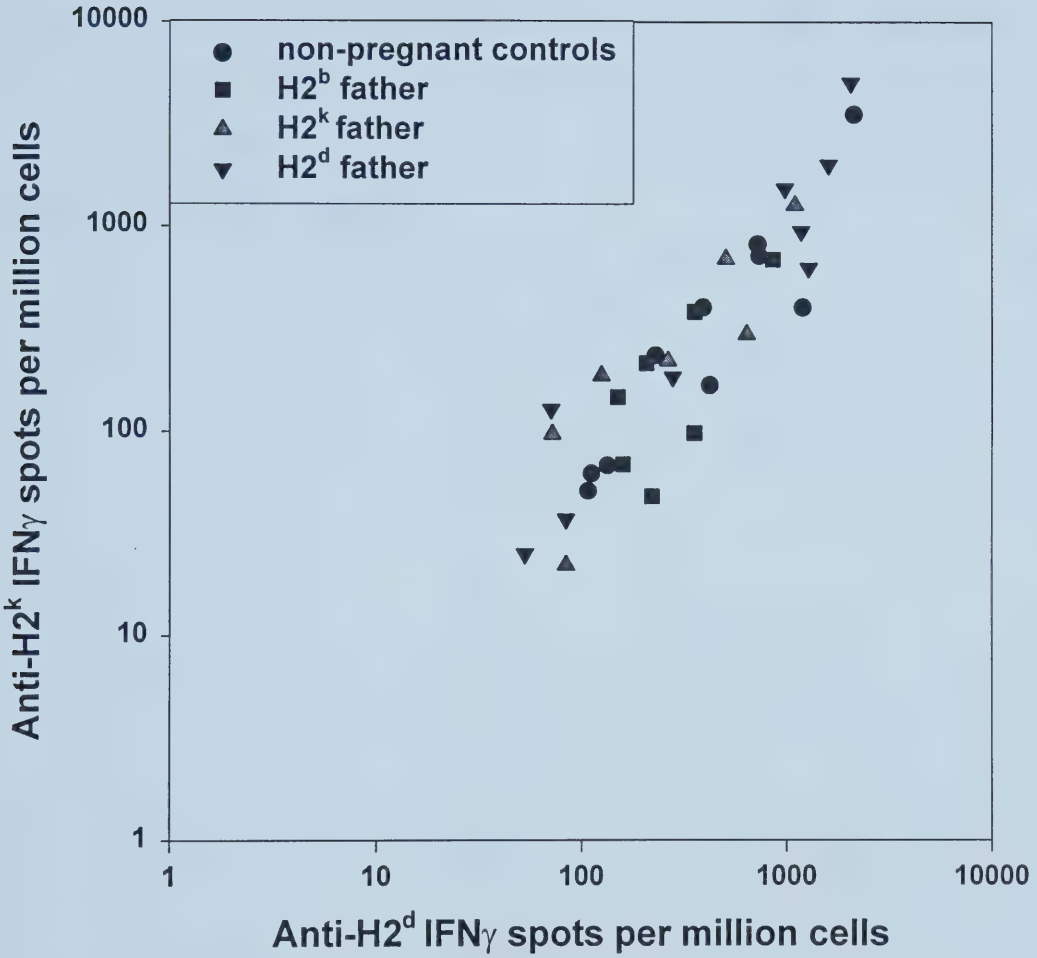

Figure 8.
Comparison of IFNγ response against $H2^k$ and $H2^d$ antigens in the same mouse after alloimmunization.
C57BL/10 ($H2^b$) mice were mated with C57Bl/10 ($H2^b$), B10.BR ($H2^k$) or B10.D2 ($H2^d$) males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with two types of allogeneic (B10.BR ($H2^k$) and B10.D2 ($H2^d$)) dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, unseparated spleen cells were harvested and stimulated *in vitro* with allogeneic B10.BR ($H2^k$) or allogeneic B10.D2 ($H2^d$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice. This data was shown in figures 8-11.

In order to determine if pregnancy alone affects IFNγ production after immunization with MHC alloantigens, we compared alloimmunized syngeneically pregnant and non-pregnant matched control mice. The results in figure 9 show that the response against $H2^b$, $H2^d$, and $H2^k$ antigens was similar in these two groups, p= 0.436, 0.450, and 0.450, respectively. Therefore, pregnancy alone does not alter the response induced during pregnancy against MHC antigens.

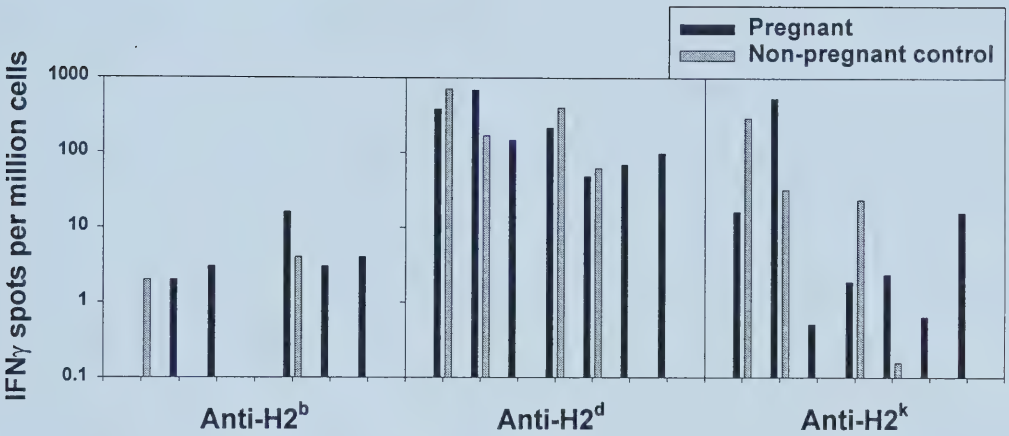

Figure 9.
IFNγ response in syngeneically pregnant and non-pregnant mice after alloimmunization. C57BL/10 ($H2^b$) mice were mated with C57BL/10 males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with two types of allogeneic (B10.BR ($H2^k$) and B10.D2 ($H2^d$)) dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, unseparated spleen cells were harvested and stimulated *in vitro* with syngeneic ($H2^b$), allogeneic B10.BR ($H2^k$), or allogeneic B10.D2 ($H2^d$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice. Adjacent black and gray bars represent pregnant and non-pregnant control mice that were used on the same day. All pregnant mice have a control mice done on the same day. In some cases, there is one control mouse per two pregnant mice. In those cases the bar for the control mouse appears adjacent to the bar of the first mice and the bar for the second pregnant mouse appears by itself.

To study if the presence of fetal alloantigens affects the response against these antigens induced during pregnancy by alloimmunization, we compared IFNγ responses in response to paternal (figure 11) and third-party antigens (figures 9 and 10) between alloimmunized mice pregnant with $H2^d$, $H2^k$, or syngeneic fetuses.

IFNγ responses against paternal antigens after immunization were similar in pregnant mice and their non-pregnant controls regardless of the paternal strain (figure 11).

In addition, anti-paternal responses were similar to anti-third party responses after immunization with both antigens, indicating that the presence of fetal alloantigens did not alter anti-paternal responses (figures 10 and 11). IFNγ responses against $H2^d$ in mice pregnant with fetuses bearing $H2^d$ antigens were similar to responses in mice pregnant with syngeneic fetuses or fetuses bearing $H2^k$ antigens ($p=0.427$ and $p=0.397$). In addition, IFNγ responses against $H2^k$ in mice pregnant with fetuses bearing $H2^k$ antigens were similar to responses in mice pregnant with syngeneic fetuses or fetuses bearing $H2^d$ antigens ($p=0.482$ and $p=0.491$). This suggests that there is no enhancement or suppression of anti-paternal MHC IFNγ responses induced during pregnancy.

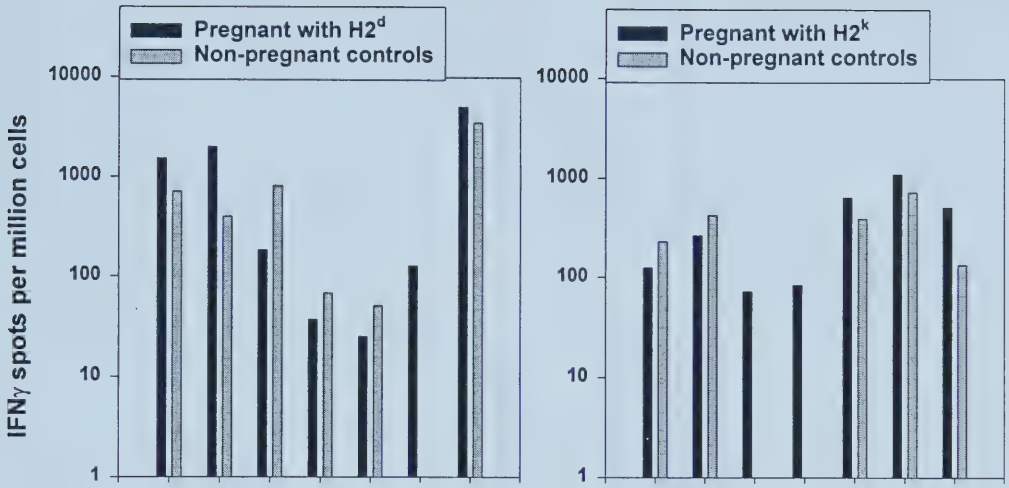

Figure 10.
IFNγ response against paternal antigens after immunization.
C57BL/10 ($H2^b$) mice were mated with B10.BR ($H2^k$) or B10.D2 ($H2^d$) males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with two types of allogeneic (B10.BR ($H2^k$) and B10.D2 ($H2^d$)) dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, unseparated spleen cells were harvested and stimulated *in vitro* with syngeneic ($H2^b$), or paternal allogeneic ($H2^k$ or $H2^d$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice. Adjacent black and gray bars represent pregnant and non-pregnant control mice that were used on the same day. All pregnant mice have a control mice done on the same day. In some cases, there is one control mouse per two pregnant mice. In those cases the bar for the control mouse appears adjacent to the bar of the first mice and the bar for the second pregnant mouse appears by itself.

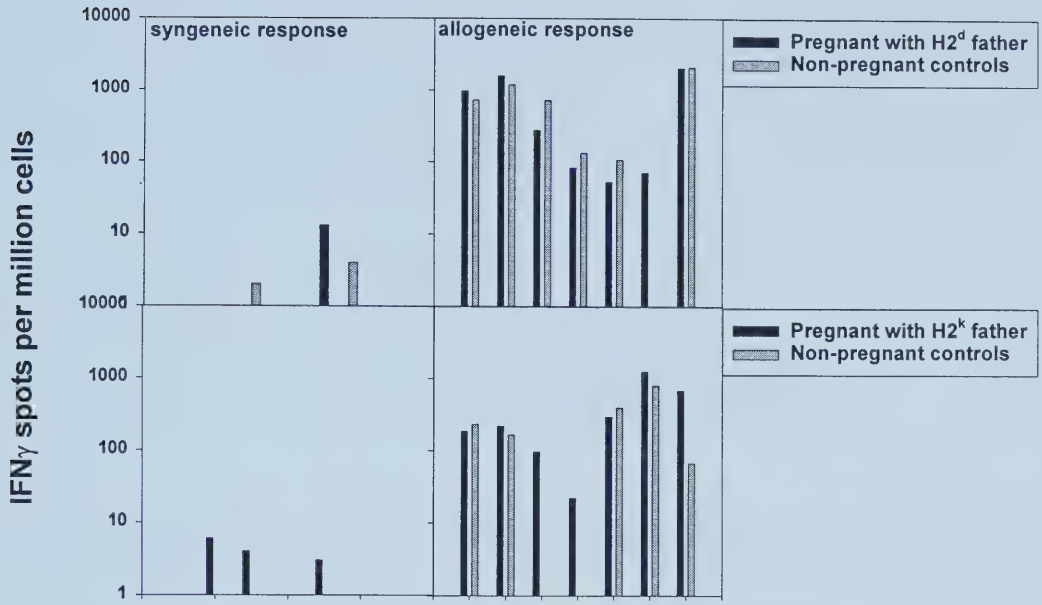

Figure 11.
IFNγ response against non-paternal antigens after alloimmunization.
C57BL/10 ($H2^b$) mice were mated with B10.BR ($H2^k$) or B10.D2 ($H2^d$) males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with two types of allogeneic (B10.BR ($H2^k$) and B10.D2 ($H2^d$)) dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, unseparated spleen cells were harvested and stimulated *in vitro* with syngeneic ($H2^b$), or non-paternal allogeneic ($H2^d$ or $H2^k$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice. Adjacent black and gray bars represent pregnant and non-pregnant control mice that were used on the same day. All pregnant mice have a control mice done on the same day. In some cases, there is one control mouse per two pregnant mice. In those cases the bar for the control mouse appears adjacent to the bar of the first mice and the bar for the second pregnant mouse appears by itself.

A more precise analysis was done by comparing anti-$H2^d$ and anti-$H2^k$ IFNγ responses in the same mouse. The results in figure 8 showed that IFNγ production was similar regardless of whether the father was $H2^k$ or $H2^d$ ($p=0.791$). Thus, exposure to fetal MHC antigens had no measurable effect on the maternal IFNγ response after immunization with paternal and third-party dendritic cells. This suggests that there is no antigen-specific suppression of IFNγ responses induced during pregnancy.

Contribution of $CD4^+$ and $CD8^+$ T cells to the allospecific IFNγ response.

As discussed in the introduction, previous experiments showed antigen-specific suppression of cytotoxicity from $CD8^+$ cells during pregnancy (43) and others showed non-antigen specific suppression of $CD4^+$ IFNγ production in syngeneic pregnancies. Because of this, we wanted to test if either $CD4^+$ or $CD8^+$ cells showed a suppression of IFNγ responses in allogeneic pregnancies, or if, in contrast, they were involved in the high response seen after immunization in our study. We separated $CD4^+$ and $CD8^+$ cells because measurement of IFNγ production with unseparated cell preparations may mask effects on one of the cell types.

In order to see if $CD4^+$ or $CD8^+$ cells were involved in the IFNγ response seen after immunization, pregnant $H2^b$ mice by syngeneic, $H2^k$ or $H2^d$ fathers were immunized at day 7 of pregnancy with a combination of $H2^k$ and $H2^d$ dendritic cells. Spleens were harvested on day 14 of pregnancy and $CD4^+$ and $CD8^+$ cells were positively sorted from

aliquots of spleen. IFNγ secretion against $H2^b$, $H2^d$, and $H2^k$ antigens was measured by ELISPOT.

After alloimmunization, IFNγ production from sorted $CD4^+$ and $CD8^+$ cells was easily measurable and variable. However, $CD8^+$ cells showed a higher response than $CD4^+$ cells (compare figures 12 and 13). As seen before for responses of unseparated spleen cells, comparison of anti-paternal and anti-third party responses in the same mouse correlated well, indicating that environmental priming affected both responses in a similar manner.

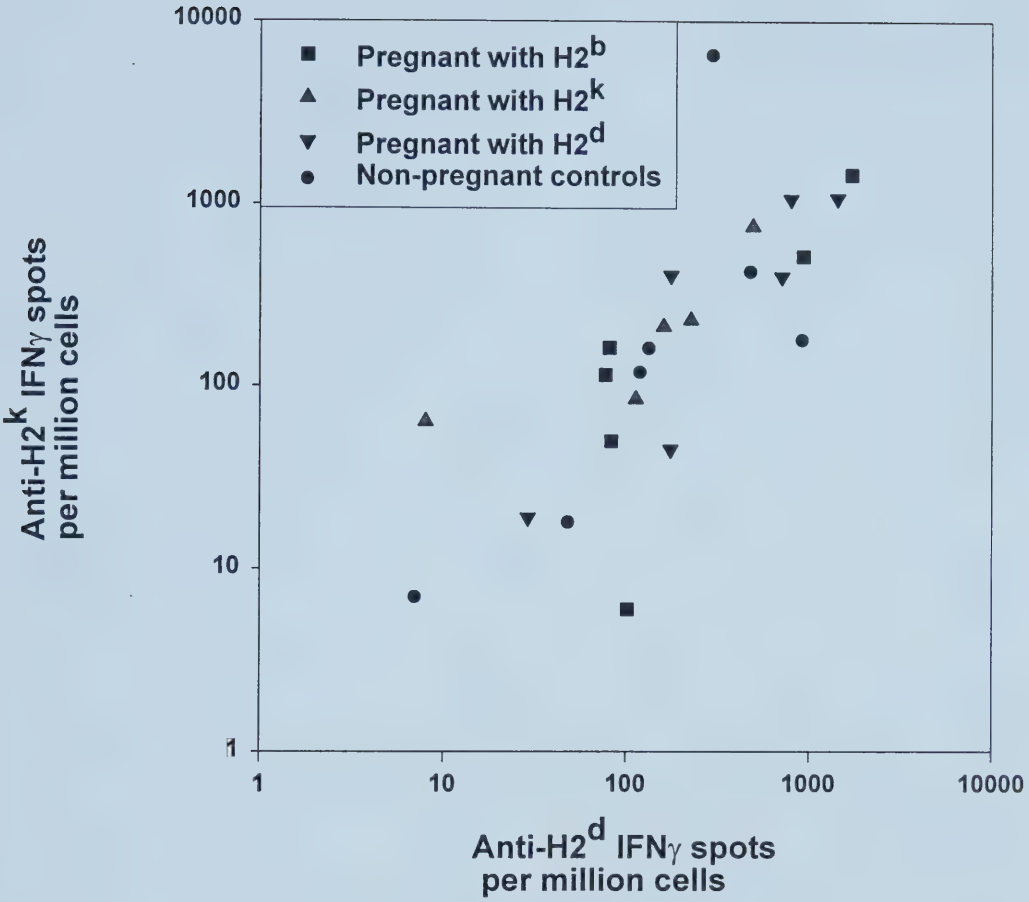

Figure 12.
$CD4^+$ IFNγ response after alloimmunization.
C57BL/10 ($H2^b$) mice were mated with C57BL/10 ($H2^b$), B10.BR ($H2^k$) or B10.D2 ($H2^d$) males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with two types of allogeneic (B10.BR ($H2^k$) and B10.D2 ($H2^d$)) dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, $CD4^+$ cells were positively sorted from aliquots of spleen and stimulated *in vitro* with syngeneic ($H2^b$), allogeneic B10.BR ($H2^k$), or allogeneic B10.D2 ($H2^d$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice. Horizontal bars represent medians.

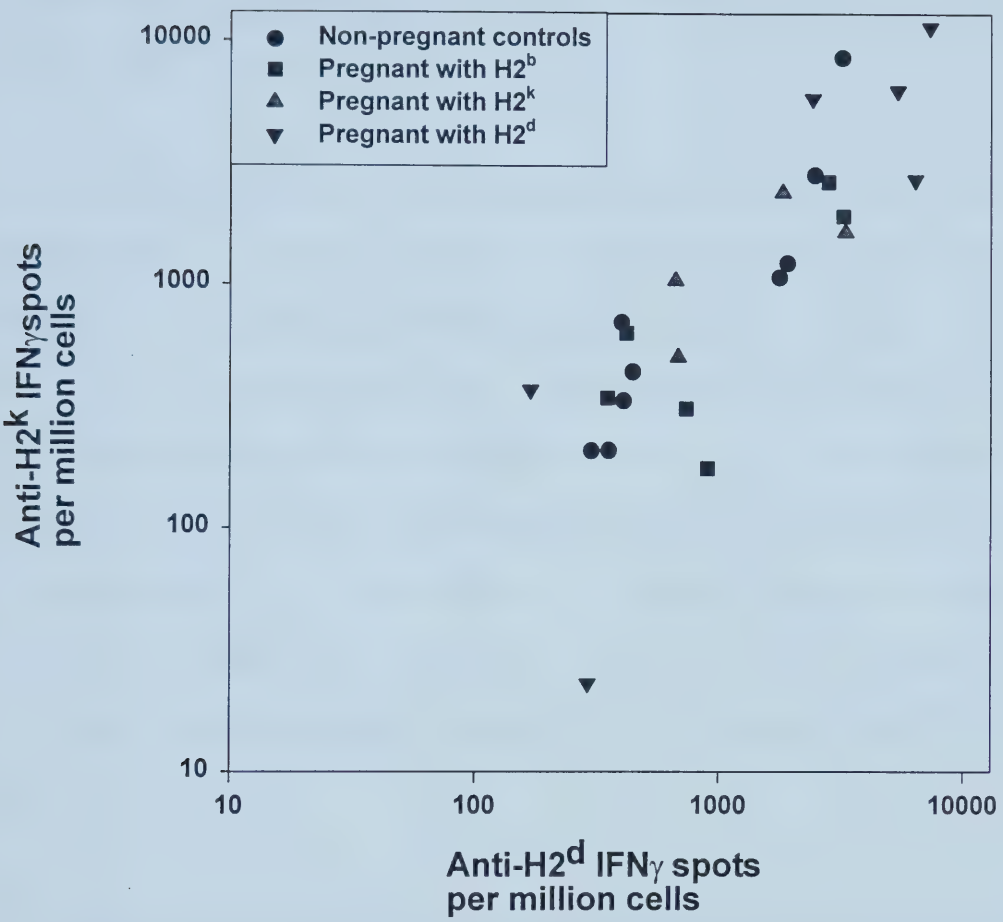

Figure 13.
$CD8^+$ IFNγ response after alloimmunization.
C57BL/10 ($H2^b$) mice were mated with C57BL/10 ($H2^b$), B10.BR ($H2^k$) or B10.D2 ($H2^d$) males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with two types of allogeneic (B10.BR ($H2^k$) and B10.D2 ($H2^d$)) dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, $CD8^+$ cells were positively sorted from aliquots of spleen and stimulated *in vitro* with syngeneic ($H2^b$), allogeneic B10.BR ($H2^k$), or allogeneic B10.D2 ($H2^d$) spleen cells (devoid of $CD4^+$, $CD8^+$, and NK cells). IFNγ production was assayed by ELISPOT. Values represent IFNγ spots per million cells from individual mice.

No enhancement or suppression of $CD4^+$ or $CD8^+$ IFNγ responses against non-paternal MHC antigens

Comparison of syngeneically pregnant and non-pregnant mice showed that responses between these two groups were similar ($CD4^+$: p=0.723 and 0.555 and $CD8^+$: p=0.556 and 0.637 for anti-$H2^d$ and anti-$H2^k$, respectively), indicating that there was no suppression or enhancement against MHC alloantigens that were NOT present on the fetus. In addition, IFNγ production by $CD4^+$ (12) and $CD8^+$ (figure 13) cells against third party antigens was also similar in mice pregnant with B10.BR (H2k) males and non-pregnant mice ($CD4^+$: p=0.739 and $CD8^+$: p=0.730), and in mice pregnant with B10.D2 males and non-pregnant mice ($CD4^+$: p=0.699 and $CD8^+$: p=0.634). This suggests that there was no general suppression or enhancement of IFNγ responses from $CD4^+$ and $CD8^+$ T cells.

No enhancement or suppression of $CD4^+$ or $CD8^+$ IFNγ responses against MHC alloantigens present on the fetus

Anti-paternal IFNγ production by both $CD4^+$ (figure 12) and $CD8^+$ (figure 13) cells was similar in pregnant mice with B10.BR males and their non-pregnant control mice (CD4+: p=0.317 and CD8+: p=0.947), and in pregnant mice with B10.D2 males and their non-pregnant controls (CD4+: p=0.630 and CD8+: p=0.491). Therefore, pregnancy does

not induce enhancement or suppression of antigen-specific $CD4^+$ T cell responses or of antigen-specific $CD8^+$ T cell responses.

In addition, as seen for responses of unseparated spleen cells, $CD4^+$ (figure 12) and $CD8^+$ (figure 13) anti-paternal and anti-third party IFNγ responses were similar in pregnant mice regardless of the paternal strains ($CD4^+$: p=0.935 and $CD8^+$: p=0.768 for anti-$H2^d$ responses, and $CD4^+$: p=0.769 and $CD8^+$: p=0.659 for anti-$H2^k$ responses). And comparison of anti-paternal and anti-third party responses from $CD4^+$ and $CD8^+$ cells in the same mouse correlated well. This suggests that there is no antigen-specific suppression or enhancement of IFNγ responses from $CD4^+$ and $CD8^+$ cells.

Thus, both $CD4^+$ and $CD8^+$ T cells were involved in the high IFNγ response seen after immunization.

Alloimmunization with paternal dendritic cells does not reduce fertility

In order to see if immunization had an effect on pregnancy, the numbers of fetuses and resorptions were compared in mice immunized with syngeneic or allogeneic cells in figure 14. Alloimmunization seemed to have no deleterious effect on pregnancy because the number of fetuses in these two groups was not significantly different (p=0.982).

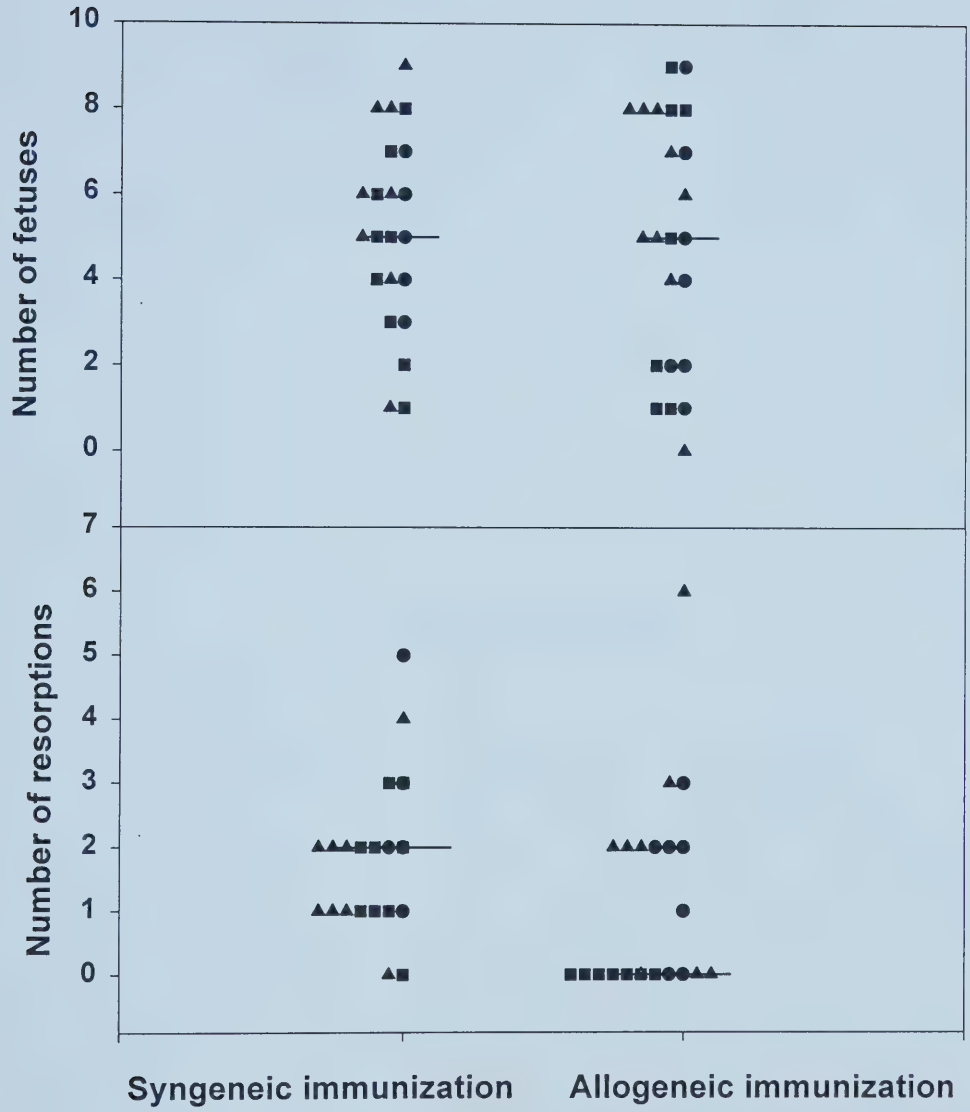

Figure 14.
Allogeneic immunization with dendritic cells does not have a deleterious effect on the number of fetuses. C57BL/10 ($H2^b$) mice were mated with C57BL/10 ($H2^b$) (●), B10.BR ($H2^k$) (■) or B10.D2 ($H2^d$) (▲) males. Pregnant (day 7 of pregnancy) and non-pregnant controls were injected i.p. with syngeneic C57BL/10 ($H2^b$), or two types of allogeneic (B10.BR ($H2^k$) and B10.D2 ($H2^d$)) dendritic cells. One spleen equivalent of dendritic cells was injected per mouse. On day 14 of pregnancy, the uterus was harvested and the number of fetuses and resorptions were counted. Values represent numbers of fetuses and resorptions from individual mice. Horizontal bars indicate medians.

DISCUSSION

A. Brief summary of results

In summary, our results show that:

1. Pregnancy alone did not enhance or suppress $CD4^+$, $CD8^+$, or unseparated spleen cell IFNγ responses against non-paternal or paternal MHC antigens.
2. An IFNγ response from $CD4^+$, $CD8^+$, and unseparated spleen cells against MHC alloantigens can be induced during pregnancy by immunization.
3. Pregnancy did not affect $CD4^+$, $CD8^+$, or unseparated spleen cell IFNγ responses induced during pregnancy against paternal or non-paternal MHC antigens.
4. Alloimmunization did not affect the number of fetuses at day 14.

Therefore, there was no evidence for pregnancy-induced specific or general suppression or enhancement of IFNγ responses against MHC alloantigens.

B Studies consistent with our findings

Our results are consistent with an earlier study (66) that used tumor protection and cytotoxicity assays to evaluate anti-paternal MHC immune responses. Normal pregnant mice mounted responses against paternal MHC antigens after injection of cells from the paternal strain (66). Tumor cells bearing paternal antigens were killed in mice immunized

with paternal cells, although this could have been due to either antibody or CTL responses. More definitive data on the priming of a $CD8^+$ T cell response was provided by the *in vitro* cytotoxicity results showing that a normal CTL response could be primed by alloimmunization during pregnancy (66). Thus pregnant mice appear to be able to mount $CD8^+$ T cell responses against paternal MHC antigens as measured by either cytotoxicity (66) or IFNγ production (this study).

C. Studies inconsistent with our findings

a) Studies in transgenic mice showing a suppression of T cell responses during pregnancy

In contrast, two recent studies using transgenic mice have shown that during pregnancy there is suppression or deletion of maternal $CD8^+$ T cells specific for paternal MHC antigens (43). In the first study, pregnant mice expressing a transgenic T cell receptor that recognized $H2^b$ antigen had a reduced proportion of T cells with high transgenic TCR expression only if the fetus expressed $H2^b$ antigens. Tumor grafts bearing the paternal antigens present on the fetus were accepted by pregnant mice. However, third party grafts were rejected (43). These *in vivo* experiments show that the presence of the fetus induces a state of tolerance for fetal antigens, as measured by tumor killing *in vivo*. In the second study, transgenic mice with a high frequency of T cells specific for the minor antigen H-Y were used. Transgenic pregnant mice showed a decreased number of T cells specific for H-Y fetal antigens. The remaining specific cells were unresponsive to antigenic stimulation in an *in vitro* cytotoxicity assay (44). These results suggest that

during pregnancy, transgenic T cells change phenotypically to avoid the rejection of the fetus.

Reasons for the difference between results in transgenic mice and our results

The different outcomes of response in the experiments presented in this thesis and the results using transgenic mice may be due to the following differences between these studies.

1) Much higher frequency of specific T cells in transgenic mice.

Transgenic mice contain a much higher frequency of allospecific cells than the normal mice used for our study. Such a large difference in frequency may affect the way in which the T cells behave. Fas dependent mechanisms have important functions in down-regulating immune responses. FasL is expressed on activated T cells and upon ligation to Fas causes the apoptosis of the Fas bearing cell. Activated allospecific T cells in transgenic mice may be too many and too close to each other due to their high frequency and may induce apoptosis of neighboring T cells through a Fas dependent mechanism. This may dampen the response to alloantigens.

2) Same T cell receptor in the whole T cell population of transgenic mice

In addition, in the transgenic animals, the avidity of the T cell receptor for antigen is high and the same in all or the vast majority of responding T cell population. In normal

mice, the avidity of the T cell receptor is varied and this may modulate the response. T cells are triggered when the TCR recognizes and engages ligands presented in the context of MHC. The amount of ligand required to activate T cells varies depending on several factors that include the state of the T cell (naïve or memory), the number of costimulatory molecules on the APC, and the ability of the TCRs to be sequentially triggered(68). T cells are sensitive to the number of triggered TCRs and respond when this number reaches an appropriate threshold. The number of TCRs engaged at one time is not capable of sustaining a signaling cascade and sequential recruitment and triggering of TCRs is necessary to maintain a signal (69). In normal mice, the TCR-peptide/MHC interaction has a very high off-rate (half-life of 4 seconds to several minutes, with a low affinity of 1μM or less) (70). This allows a single peptide/MHC complex to dissociate from the engaged TCR after triggering it, making it possible to engage and trigger several TCRs sequentially. In transgenic mice, the affinity of all TCRs for antigen is very high and this may interfere with their capacity to dissociate and allow serial triggering, affecting the response of the whole T cell population. It may be easier to suppress responses in transgenic pregnant mice because of the low off-rates in the TCR-peptide/MHC interaction.

3) Selective suppression of effector functions in transgenic animals

It is also possible that only certain aspects of T cell function are suppressed during pregnancy in transgenic mice. The transgenic studies measured cytotoxicity *in vitro* and

tumor killing *in vivo*. The results presented in this paper measured IFNγ production *ex vivo*.

4) Transgenic mice may lack normal regulation

Because the T cell repertoire is fixed in transgenic mice, it is not possible to study any effect of repertoire diversity or clonal maturation in these mice. In addition, cell to cell interactions that result from TCR diversity do not occur and this may distort the establishment of certain effector regulatory functions. Therefore, responses to infection in transgenic mice might be different due to the lack of normal T cells. Transgenic TCRs are derived from mature lymphocytes that have been carefully selected. They are then expressed in developing lymphocytes that do not express receptors present in mature cells. This precocious expression of TCRs could have a strong and abnormal effect on the development of the transgenic cells (71).

It is also possible that infectious in transgenic mice may lead to higher inflammation than in normal mice because the repertoire of TCRs is very low and they may not be able to deal with infections as effectively as normal mice.

b) Conflict with studies showing a pregnancy-induced non-specific IFNγ suppression

Our present results show that during allogeneic pregnancy, there is no non-specific suppression of IFNγ responses. This seems to contrast with previous studies showing a non-specific suppression of IFNγ responses in pregnant mice (42). In C57Bl/6 mice

infected with Leishmania major, both pregnant and non-pregnant mice mounted IFNγ responses, as measured by stimulation of spleen cells with parasite antigens. However, the pregnant mice had significantly lower responses. These results were interpreted in terms of a model (72) that maternal cytokine patterns during pregnancy shift away from a Th1 response in order to protect the fetus because inflammatory and cell-mediated responses are harmful to the fetus (47;73). These experiments were done using syngeneic pregnancies only. In our study, comparison of syngeneically pregnant, and allogeneically pregnant mice with non-pregnant matched controls showed that IFNγ production was similar in both groups. This indicates that there was no pregnancy induced non-specific suppression of IFNγ in response to MHC antigens.

Possible reasons for discrepancy with anti-Leishmania IFNγ response:

1. Higher frequency of allospecific T cells than Leishmania-specific T cells.

This apparent discrepancy may be due to a much higher frequency of allospecific T cells than for Leishmania antigen-specific T cells. Responses to MHC differ greatly from responses to other antigens because MHC alloantigens present peptides themselves. This allows the formation of thousands of different allogeneic combinations of MHC/peptides, which can stimulate a high frequency of T cells (4). Therefore, there is a high allospecific response even in naïve mice (approximately 1/20 T cells will respond to alloantigens). In contrast, the frequency of T cells specific for Leishmania major antigens is much lower (approximately $1/10^5$cells).

In addition, background MHC responses are variable due to environmental priming, and small differences in the response may be obscured. In our study, the difference in IFNγ production by pregnant and non-pregnant mice may have been too small to measure at day 14 of pregnancy due to the small increase in frequency of allospecific T cells. However, our preliminary experiments using minor histocompatibility antigen differences between mother and fetuses (data not shown) show no pregnancy-induced IFNγ suppression against these antigens. Responses to minor histocompatibility antigens are more similar to anti-Leishmania responses because in both cases T cells specific for these antigens are present in low frequency.

2. Site of priming: footpad and draining lymph node, versus peritoneal cavity and draining lymph node.

A second important difference between our experiments and the experiments using Leishmania infection during pregnancy is the site of antigen priming. T cells are primed for Leishmania antigens in the draining lymph node of the footpad. In our experiments, we injected allogeneic dendritic cells intraperitoneally; therefore, T cells are primed in the draining lymph node of the peritoneal cavity. Previous experiments have shown that the site and form of antigen administration may determine induction of memory or tolerance for the antigen (74).

D. Lack of IFNγ priming during pregnancy

Our results show that there was no pregnancy induced enhancement or suppression of IFNγ responses against MHC antigens. However, we were able to induce a strong anti-MHC IFNγ response after deliberate immunization with two types of allogeneic dendritic cells during pregnancy. The reason for this is not clear, but several factors may contribute to this outcome.

a) Differences in antigen presentation in pregnancies with and without deliberate alloimmunization

Quantitative and qualitative differences between the fetal cells presenting paternal antigens during normal pregnancy and the dendritic cells that we used for immunization may explain the lack of IFNγ priming during pregnancy with no deliberate immunization and the increase of IFNγ response after immunization with paternal dendritic cells.

We chose to immunize with dendritic cells because they initiate and modulate immune responses by efficiently capturing antigens in the periphery and processing them to form MHC/peptide complexes. The type of T cell response stimulated by the antigen presenting cell depends on mode of antigen internalization, the costimulatory molecules on the dendritic cell, and the cytokine milieu in which the APC/T cell interaction takes place. In our study, antigen presentation without and with alloimmunization differ greatly and this may explain the lack of enhancement or suppression in the first case and the higher response seen in the second case.

For immunization with paternal antigens we used a very high number of dendritic cells (0.5 spleen equivalents or approximately $0.5x10^6$ cells). In addition, the dendritic

cells used for immunization in our experiments may be qualitatively different to the ones presenting fetal antigens in normal pregnancies. For example, the latter could express high levels of Fas and other death-inducing molecules or they could secrete cytokines, such as TGF-β, to selectively dampen cytotoxic responses.

The data presented here indicate that pregnancy did not strongly prime an IFNγ response against paternal antigens. This may mean that fetally derived cells are not immunogenic, or that the placenta acts as an impermeable barrier between the fetal and maternal compartments.

b. Placental barrier

The placenta and fetal membranes provide an interface for the exchange of nutrients and waste products to and from the fetus and also function as a physiological barrier against infection and immune attack. In rodents three extraembryonic structures: the placenta, the visceral yolk sac, and the amnion surround the embryo (75). Trophoblast cells are a specialized cell type that contributes to the placenta. They are essential for contacting the uterine wall during implantation, invading it and producing hormones and cytokines that promote the success of pregnancy. The trophoblast acts as a semi-permeable barrier, which allows nutrients and gases through, but restricts passage of molecules and cells that may damage the fetus.

Molecules that are protective to the fetus may be transferred from the mother to the fetus in a selective manner. Transfer of maternally derived antibodies is mostly beneficial to the offspring because antibodies against invading parasites may help modulate the fetal

immune response and enhance the offspring's capacity to mount a response in a subsequent infection (76). A number of placental proteins transport IgG from the mother to the fetus (77) . Most of the antibodies present in the blood of a newborn are maternal. However, maternal humoral immune responses against the fetal foreign proteins that could potentially damage the fetus occur during pregnancy. The placenta can absorb these types of antibodies and this protects the fetus (78).

Although our results show that substantial numbers of IFNγ-producing cells can be induced *in vivo* against paternal antigens during pregnancy after deliberate immunization, this response does not result in loss of fetuses, suggesting that the allospecific cells do not reach fetal tissues, or that they are functionally suppressed in the placenta and fetus.

c. Local immune regulation at the maternal-fetal interface

The trophoblast has developed a number of mechanisms that protect fetal cells from coming into contact with activated T cells.

1. Regulation of MHC expression

The trophoblast does not express classical MHC and this hinders recognition by cytotoxic T cells (79). In humans, HLA A and B are not expressed in trophoblast (80). NK cells are present in large numbers in the pregnant uterus (81) and may damage fetal cells that do not have a normal repertoire of MHC proteins on their surface (82). In order to address this problem, the trophoblast expresses non-classical MHC molecules, such as

HLA-G (83;84) and HLA-E (85), which together inhibit NK killing (86). HLA-E interacts with a CD94/NKG2, which inhibits NK cytotoxicity (87). HLA-G may not only inhibit cytotoxicity, but may also limit transplacental migration of maternal and fetal NK cells (88).

In mouse, non-classical MHC molecules Qa-1 and Qa-2 are expressed in placenta and may function in a manner analogous to human HLA-G and HLA-E (89).

Thus, the lack of classical MHC expression and the expression of non-classical MHC on trophoblast prevents normal recognition of fetal tissue by T cells and suppresses NK killing.

2. FasL expression at the maternal-fetal interface

The fetus is also protected from attack by activated T cells by the expression of FasL on trophoblast and decidual cells. FasL is also transiently expressed on activated T cells. FasL binds to Fas, which is expressed on a variety of cells, including activated T cells. The binding of FasL to Fas causes apoptosis of the Fas-bearing cells. Trophoblast and decidual cells express FasL to kill activated T cells that cross the placenta (40).

In addition, activated T cells are down-regulated at the maternal fetal interface by the production of indolamine 2,3-dioxygenase (IDO) by placental cells. IDO is an enzyme that inhibits T cell proliferation by increasing tryptophan catabolism (90). The importance of this protective mechanism during pregnancy was shown in previous studies. If this enzyme was inhibited pharmacologically, allogeneic but not syngeneic

fetuses were rejected by activated T cells (2), indicating that allospecific T cells have the potential to destroy the fetus.

d. Immunogenicity of fetal cells

Our results show that, even when there is an induction of an IFNγ response by deliberate immunization during pregnancy, there is no damage to the fetus in mid-pregnancy. Previous studies have shown that fetal cells can enter the maternal circulation, and if allogeneic, these cells are destroyed rapidly by the maternal immune system (91) indicating that the maternal immune system can recognize and destroy allogeneic fetal cells. In this study the number of allogeneic fetal cells crossing the placenta may be very small and may not allow priming of a significant IFNγ response that could damage the fetus. Also, in normal pregnancy, the allogeneic cell may not be strongly immunogenic or it may not migrate to the appropriate lymphoid tissue.

E. Cytokine regulation in other stages of pregnancy

a. Insemination

It has been proposed that the events that lead to tolerance of the allogeneic fetus start at insemination. Semen provides antigenic and environmental signals to establish T cell tolerance to paternal alloantigens (reviewed in 92). Females first encounter paternal alloantigens in the context of semen and later encounter these antigens in the fetus. Seminal antigens include major histocompatibility antigens, MHC class I, MHC class II

(93), and minor antigens including H-Y (94)and CD46 antigens (reviewed in 92). Insemination constitutes a priming event that may start the induction of maternal tolerance to paternal alloantigens.

Introduction of male ejaculate during periovulatory period has been shown to produce an inflammatory response in mice, with an increase in inflammatory cytokines and chemokines and an influx of macrophages, dendritic cells and neutrophils (95). Infiltrating cells show an increase in antigen uptake and in the expression of costimulatory molecules (reviewed in 92). This response may promote efficient antigen presentation of seminal antigens.

Seminal plasma promotes sperm survival, transport, and persistence in the female tract by eliciting immunosuppressive activity. Semen can influence endometrial receptivity and placental development because it contains PGE-2 and TGF-β (96)in high levels that can induce a Th2 response (97). This effect is augmented by synthesis of TGF-β, PGE-2 as well as Th3 cytokines in the female tract (reviewed in 92).

Cumulative exposure to semen might strengthen the capacity of the female tract to accommodate the allogeneic fetus in a partner-specific manner. Reproductive outcome is improved is mother is exposed to seminal fluid at the outset of pregnancy (reviewed in 92). Also, exposure to semen in mice is sufficient to induce a state of specific tolerance to paternal MHC I alloantigens, even in the absence of pregnancy. Extended cohabitation also promotes pregnancy success in humans (98). This effect seems to be specific because protection is lost in women having a new partner (99). Therefore, insemination may promote activation and expansion of populations of lymphocytes that mediate immune tolerance to paternal alloantigens in a specific manner.

b. Implantation and embryo development

During implantation fetally-derived cells invade a modified layer of maternally-derived mucosal tissue lining the pregnant uterus. This process results in the intimate interaction between allogeneic fetal and maternal cells.

Successful implantation and maintenance of the embryo and fetus is also influenced by cytokine expression in the peri-implantation endometrium. Progesterone may be partly responsible for a Th2 bias in the cytokine milieau at the materno-fetal interface. IL4 can then promote the secretion of leukemia inhibitory factor (LIF) by T cells (100), which is essential for successful implantation and embryo development. Female mice that lack a functional LIF gene are can be fertilized, but show implantation failure (101).

Th1 cytokines can be detrimental to embryo development by down-regulating development of LIF producing cells (reviewed in 100). In addition, murine interferons have been reported to inhibit proliferation of mouse embryonic cells in primary cultures and both human and murine IFNγ is toxic to embryo development (102). IFNγ could inhibit embryo development and implantation by altering the expression of embryonic cell surface antigens (102) or inhibiting translational mobility of plasma membrane proteins in the preimplantation stage (103).

However, during pregnancy, type 2 cytokines play an important role in the regulation of inflammatory responses. For example, type 2 cytokines such as IL4 and IL10 inhibit the differentiation and function of type 1 cells. Therefore, the cytokine microenvironment during the implantation window prevents the rejection of the allogeneic embryo and promotes implantation and embryo development (104).

c. Placentation

In normal pregnancies inflammatory responses that may harm the placenta are modulated and inhibited. Maternal inflammatory responses during pregnancy may clear unhealthy conceptuses and may ensure a prompt spontaneous abortion of the fetus (105).

It has been suggested that preeclampsia may be caused by a generalized inflammatory reaction involving intravascular leukocytes (105).

d. Parturition and Pre-term labor

Inflammatory cytokines secreted by human term myometrium are thought to be involved in parturition. Increased concentrations of IL1, IL6, IL8, and TNFα during term and pre-term birth supports the hypothesis that parturition involves an inflammatory process. IL8 and TNFα are expressed in myometrium only during active labor, and IL-1 and IL6 concentrations increase dramatically during active parturition (106). Gene expression of IL8 is also increased during labor in humans (107). Inflammatory cytokines may promote cervical ripening by inducing the production of hyaluronic acid by cervical fibroblasts (108).

After labor, there is increased production of IL10 by decidual tissue, possibly to terminate the inflammatory response associated with labor (109).

Pre-term labor is a syndrome due to several potential insults to the uterus or the fetus. Pre-term labor may be precipitated by many factors, including inflammation of the uterus. This may be due to intrauterine infection, which is associated with inflammatory cytokines. In some cases pre-term labor caused by inflammation is not associated with an

identifiable infectious organisms and it is called "intrauterine inflammatory response syndrome" (110). IL1 (111) and IL6 (112) are present in high concentrations in amniotic fluid in women with spontaneous pre-term delivery. TNFα in the cervicovaginal fluid of women with uterine contractions is considered a predictor of pre-term delivery (113).

Therefore, evidence suggests that cytokine regulation may play a role in all stages of pregnancy, beginning with insemination and ending with resolution of labor. The results in this thesis show that, during mid-pregnancy, IFNγ secretion by T cells specific for paternal antigens is not affected by pregnancy and is compatible with a successful pregnancy.

E. Conclusions

Therefore, even if some T cell functions, such as $CD8^+$ cytotoxicity *in vitro* (114) and $CD8^+$ tumor killing *in vivo* (43), are suppressed during pregnancy in transgenic mice, other functions, such as *ex vivo* production of IFNγ by $CD4^+$ and $CD8^+$ cells, are not suppressed during pregnancy in normal mice. This indicates that IFNγ production in response to allogeneic stimulation does not damage the fetus and is compatible with a successful pregnancy.

Given that normal pregnancies are always allogeneic, it is important to understand the responses against paternal alloantigens to gain insight into the way in which the fetus evades rejection by the maternal immune response.

FUTURE EXPERIMENTS

1)

In order to measure only responses initiated during pregnancy, it would be better to study pregnancies in which the mother and fetuses differ in minor antigens than ones that differ in major histocompatibility antigens.

It is difficult to measure responses initiated during pregnancy only using mice that differ in the MHC because pre-existing responses against allogeneic minor antigens in the context of self-MHC will crossreact with responses against self or foreign proteins in the context of allogeneic MHC. This is because MHC antigens present peptides to T cells themselves.

Therefore, analysis of maternal IFNγ production in response to fetal minor histocompatibility alloantigens, using mouse strains that share identical MHC antigens but where otherwise genetically different (e.g. AKR, CBA/J, and B10.BR which share the MHC haplotype $H2^k$, but differ in the rest of the genes) is ideal to study only responses that are initiated during pregnancy.

2)

A potential problem of the experiments presented here is that they were done on different days. This was because a small percentage of mice in mating cages were actually pregnant each day. (There was always a non-pregnant control done for every pregnant mouse.) This has the potential of introducing great variability to the experiments because different cell preparations used for immunization and challenging may induce

higher or lower responses depending on the properties of these cells. In the future it would be better to do at least one mouse from each group on the same day, to avoid comparisons of experiments done on different days. In order to accomplish this, the number of mating cages would have to be increased according to percentage of plugged mice.

3)

In order to test whether our results conflict with the results in transgenic models showing suppression of T cell responses during pregnancy, adoptive transfer experiments would be useful. Transgenic mice may not have normal T cell regulation because all T cells in the mouse have developed with transgenic T cell receptor and normal regulatory functions may be altered (71). Transfer of transgenic T cells into normal mice would provide a situation where T cell regulation is more normal. In future experiments, transfer of transgenic T cells during pregnancy may be a good model to test if the suppression seen in other systems applies to the regulation of IFNγ responses. Some advantages of using this model are: (1) the existence of anti-clonotypic antibodies allows the detection of small numbers of cells, (2) purification of naïve cells before adoptive transfer is possible, (3) the presence of all other T cells contributes a more normal T cell regulation.

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